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Escola de Engenharia

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Biodegradation of PHA/PBAT packaging materials by soil microroganisms

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Miguel Ângelo Cavaleiro Fernandes
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Biodegradation of PHA/PBAT packaging materials by soil microorganisms

PhD Thesis

Chemical and Biological Engineering

Trabalho efetuado sob a orientação do(a)
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Biodegradação de materiais de embalagem PHA/PBAT por microrganismos do solo

Resumo

Nas últimas décadas, polímeros biodegradáveis têm sido estudados como uma solução promissora para superar a poluição por plástico, com novos produtos desenvolvidos no sentido de responder às questões ambientais, mantendo as propriedades necessárias para aplicações específicas. Isso inclui o uso de polímeros biodegradáveis, como polihidroxialcanoatos (PHAs), com outros polímeros, como o polibutileno adipato tereftalato (PBAT), que é compostável. Esses materiais raramente são avaliados em termos de biodegradação para identificar se de facto são mais sustentáveis. O objetivo deste trabalho foi analisar a biodegradação aeróbia de filmes bicamada PHAs/PBAT, em solo. A biodegradação aeróbica de filmes bicamada de polihidroxibutirato-co-hidroxivalerato (PHBV)/PBAT e polihidroxibutirato (PHB)/PBAT (27 °C), atingiu 46 ± 3 % em 7 meses e 47 ± 1 % em 6 meses, respetivamente. Todas as análises indicaram que a camada PHBV (ou PHB) desapareceu dos resíduos finais. O PBAT contribuiu para os resultados com claros sinais de biodegradação. Dois fungos isolados e identificados como espécies de *Aspergillus* e três espécies, estreitamente relacionadas com *Streptomyces coelicoflavus, Clonostachys rosea* e *Aspergillus insuetus* foram indicados pela primeira vez como degradadores de PHBV ou PHB, respetivamente. Notavelmente, dois fungos estreitamente relacionados com *Purpureocillium lilacinum* e *Aspergillus pseudodeflectus* foram isolados e identificados como degradadores de PBAT. A decomposição do filme PHBV/PBAT, provocou a alterações significativas na diversidade taxonómica da comunidade microbiana. Foi também testada a influência de 3 fatores abióticos principais na biodegradação do filme PHB/PBAT. A abordagem baseada num Desenho Rotacional Composto Central revelou que temperaturas mais altas (37 °C < 45 °C) melhoraram a biodegradação e 60 % da capacidade de retenção de humidade produziu resultados semelhantes aos recomendados pelas normas oficiais. Foi desenvolvido um modelo de previsão da biodegradação do filme PHB/PBAT para solos e condições idênticas, que gerou resultados reprodutíveis. Finalmente, monoculturas e co-culturas dos 2 degradadores mesofilos de PBAT com filmes PHB/PBAT ou PBAT confirmaram que ambos degradam PBAT, mas degradam preferencialmente PHB. Os resultados, forneceram informações sobre a biodegradação dos filmes no solo, demonstrando o seu potencial para substituir plásticos convencionais, e revelaram a importância de diferentes fatores abióticos no processo. Embora o PBAT seja mais difícil de biodegradar, os dois fungos descobertos, abrem a oportunidade a novas estratégias de biodegradação no local ou fora dele.

Palavras-chave: Biodegradação aeróbica, Desenho Rotacional Composto Central, Microbioma do solo Polibutileno adipato tereftalato, Polihidroxialcanoatos

Biodegradation of PHA/PBAT packaging materials by soil microorganisms

Abstract

In the last few decades, biodegradable polymers have been studied as a promising solution to overcome plastic pollution, with new products being developed in the prospect of replying to environmental issues while preserving the required properties for specific applications. This includes using biodegradable polymers such as polyhydroxyalkanoates (PHAs) with other polymers such as polybutylene adipate terephthalate (PBAT) that is compostable. These materials are rarely evaluated in terms of biodegradation to identify if in fact are more sustainable than conventional plastics. This work aimed to analyse the aerobic biodegradation in soil of PHAs/PBAT bilayer films. In the first two steps the aerobic biodegradation of polyhydroxybutyrate-co-hydroxyvalerate (PHBV)/PBAT and polyhydroxybutyrate (PHB)/PBAT films (27 °C), reached 46 ± 3 % in 7 months and 47 ± 1 % in 6 months, respectively. All the properties analyses indicated that the PHBV (or PHB) layer was not present in the final residues. The PBAT also contributed to the results with clear signs of biodegradation. Two fungi isolated and identified as *Aspergillus* species and three species, closely related to *Streptomyces coelicoflavus*, *Clonostachys rosea* and *Aspergillus insuetus* were indicated for the first time as PHBV or PHB degraders, respectively. Most remarkably, two fungi closely related to *Purpureocillium lilacinum* and *Aspergillus pseudodeflectus* were isolated and identified as PBAT degraders. The decomposition of the PHBV/PBAT film caused significant changes in the taxonomic diversity of the microbial community. The influence of three main abiotic factors was also tested on the biodegradation of the PHB/PBAT film. The Central Composite Rotational Design approach revealed that higher temperatures (37 °C < 45 °C) improved biodegradation, and 60 % moisture holding capacity produced similar results to the recommended range by official standards (80 – 100 %). A prediction model was also developed for the biodegradation of this film in similar soils and conditions, generating reproducible results. Finally, monocultures and coculture of the two PBAT mesophilic degraders, with PBAT or PHB/PBAT films confirmed that both are capable of degrading PBAT but degrade preferably PHB. The results provided insights into the biodegradation of the bilayer films in soil demonstrating its potential to replace synthetic plastics while revealing the importance of different abiotic factors on the process. Although PBAT was more difficult to biodegrade, 2 fungi were found to degrade PBAT, opening the opportunity for the development of new in-situ or ex-situ biodegradation strategies.

Keywords: Aerobic biodegradation, Central Composite Rotational Design, Polybutylene adipate terephthalate, Polyhydroxyalkanoate, Soil microbiome

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LIST OF GENERAL NOMENCLATURE

Abbreviations

3HHx - 3-hydroxyhexanoate

4HB - 4-hydroxybutyrate

AA- - Anhydride-treated

AFM - Atomic force microscopy

AgNP - Silver nanoparticles

ANOVA - One-way analysis of variance

ASTM - American Society for Testing and Materials

ATR-FTIR - Attenuated total reflectance - Fourier-transform mid-infrared spectroscopy

bio-PA - Bio-based Polyamide

bio-PBS - Bio-based Polybutylene Succinate

bio-PE - Bio-based Polyethylene

bio-PE - Bio-based Polyethylene

bio-PET - Bio-based Polyethylene terephthalate

bio-PET - Bio-based Polyethylene Terephthalate

bio-PP - Bio-based Propylene

bio-PTT - Bio-based polytrimethylene terephthalate

BOD - Biochemical oxygen demand

C:N ratio - Carbon:nitrogen ratio

CAB - Cellulose acetate butyrate

CCRD - Central Composite Rotational Design

CEN - European Committee for Standardization

CfCLE - Enzyme from the yeast *Cryptococcus flavus* GB-1

CNT - Carbon nanotubes

ddNTPs - Dideoxynucleotide triphosphates

DGGE - Denaturing gradient gel electrophoresis

DMR - Direct measurement respirometry

DoE - Design of experiments

DSC - Differential scanning calorimetry

F0 - Olive pomace crude pomace

FISH - Fluorescent in situ hybridization

FsC - Cutinase from *Fusarium solani*

FTIR - Fourier transform infrared spectroscopy

GC - Gas chromatography

GPC - Gel permeation chromatography

HiC - Cutinase from *Humicola insolens*

HMMs - Hidden Markov Models

HPLC - High-performance liquid chromatography

ISO - International Standardization Organization

ITS - Internal Transcribed Spacer

LC - Liquid chromatography

LDPE - Low-density Polyethylene

McI - PHA - Medium-chain-length polyhydroxyalkanoates

MEG - Renewable ethylene glycol

MHC - Soil moisture-holding capacity

MIR - Mid-infrared

MIRS - Mid-infrared spectroscopy

MS - Mass spectroscopy

NDIR - Nondispersive infrared sensor

NGS - Next-generation sequencing

NIR - Near infrared

NMR - Nuclear magnetic resonance

OMW - Olive mill wastewater

P4HB - Poly(4-hydroxybutyrate)

PBAT - Polybutylene adipate terephthalate

PBATHRF - Hydrolase from *Rhodococcus fascians* NKCM2511

PBS - Polybutylene succinate

PBSA - Polybutylene succinate adipate

PBSe - Polybutylene sebacate

PBSeT - Polybutylene terephtalate co-sebacate

PCA - Principal component analysis

PCL - Poly(ε-caprolactone)

PCLE - Cutinase from *Paraphoma* sp- B47-9

PCR - Polymerase chain reaction

PDLLA - Polyd,l-lactide)

PE - Polyethylene

PEF - Polyethylene Furanoate

PEG - Poly(ethylene glycol)

PEO - Polyethylene oxide

PET - Polyethylene terephthalate

PF - Palm fiber

PF - Olive pomace pulp-rich fraction

PGA - Polyglycolic acid

PHA - Polyhydroxyalkanoate

PhaA - 3-ketothiolase

PhaB - Acetoacetyl-CoA

PhaC - PHB synthase

PHA-g-AA - Acrylic acid-grafted polyhydroxyalkanoate

PHA-g-MA - Maleic anhydride-grafted polyhydroxyalkanoate

PHAs - Polyhydroxyalkanoates

PHB - Polyhydroxybutyrate

PHB3HHx - Polyhydroxybutyrate-co-hydroxyhexanoate

PHB-g-AA - Acrylic acid-grafted polyhydroxy butyrate

PHBV - Polyhydroxybutyrate-co-hydroxyvalerate

PHH - Polyhydroxyhexanoate

PHO - Polyhydroxyoctanoate

PHV - Polyhydroxyvalerate

PLA - Polylactic Acid

PLA/PHB_b 90D - PLA/PHB outdoor weathering foil exposed 90 days to sunlight

PP - Polypropylene

PP-co-PE - Polypropylene-co-polyethylene

PP-g-MA - Graphitized polypropylene with maleic anhydride

PVA - Polyvinyl alcohol

rRNA - ribosomal RNA

scl-PHA - Short-chain-length polyhydroxyalkanoate

SDS - Sodium dodecyl sulphate

SEM - Scanning electron microscopy

SF - Olive pomace stone-rich fraction

SMG - Styrene-maleic-anhydride-glycidyl methacrylate

t – TPF - Treated crosslinked) tea plant fiber

TCA - Tricarboxylic acid cycle

TEM - Transmission electron microscopy

TGA - Thermogravimetric analysis

TPF - Tea plant fiber

TPLF - Coupling agent-treated palm fiber

TPS - Thermoplastic starch

T-RFLP - Terminal restriction fragment length polymorphism

WF - Wood flour

XPS - X-ray photoelectron spectroscopy

XRD - X-ray diffraction spectroscopy

Symbols:

ΔH - Melting enthalpy

ΔH0 - Melting enthalpy for 100 % crystalline polymer

t - time (months)

T_c - Crystallization temperature

T_d - Decomposition temperature

T_g – Glass transition temperature

T_m - Melting temperature

X_c - Crystallinity

x_1 – C:N ratio

x_2 - Temperature

x_3 - Moisture holding capacity

y - Film biodegradability

CHAPTER 1.
GENERAL INTRODUCTION

1.1 RESEARCH BACKGROUND AND MOTIVATION

Plastics have become crucial for modern society. Their specific characteristics, including durability, processability, and low production price have led to their extensive use in varied applications worldwide since the middle of the twentieth century (Trinh Tan et al., 2008). Nonetheless, their resistance led to the persistence in the environment, as trash on land, and in drainage systems, eventually leaking into rivers and oceans (Pathak et al., 2014). An increasing amount is also ending up in landfills as part of municipal solid waste, with a considerable amount ending up contaminating the soil (Pathak et al., 2014). It is necessary to create worldwide waste management infrastructures to recover and recycle plastics. However, there are difficulties in plastic recovering and recycling, due to several factors, such as plastic contamination with for example food residues (Flury and Narayan, 2021).

Consumers are becoming more aware of global plastic pollution and with society awakening, the pressure and demand on the responsible entities as well as on companies, to find sustainable alternatives, and tackle the plastic crisis reached levels never seen before. The solution involves a multifaceted approach concerning prevention, reuse, recycling, recovery, and disposal. Within this policy, biodegradable plastics are key elements. Biodegradable plastics may offer more end-of-life routes (composting and anaerobic digestion) than the more resistant conventional plastics, while also being decomposed by microorganisms without harmful effects on the environment (Gómez and Michel, 2013). These polymers can be even more interesting from an environmental point of view when their origins are natural resources or biomass (bio-based) and not petrochemical resources (Gómez and Michel, 2013). In this group, a family of polymers that stands out are the polyhydroxyalkanoates (PHAs), which are produced by many microorganisms and accumulate intracellularly as energy and carbon storage compounds (Cruz et al., 2016). They are easily biodegradable in different environments (i.e., landfill, soils, sewage sludge) by several types of bacteria and fungi capable of decomposing them (Boyandin et al., 2012; Nishida and Tokiwa, 1993; Sang et al., 2002). Furthermore, PHAs have different properties depending on their composition and can demonstrate thermoplastic or elastomeric properties, which are relevant properties for many applications including agriculture and horticulture, drug- delivery agents and packaging (Ashby and Solaiman, 2008). Unfortunately, similarly to other biodegradable polymers, PHAs properties are not enough to fulfil the purpose of most plastic applications, or is not possible to use them alone, due to their low processability in industrial settings (Teixeira et al., 2020). The use of these plastics with other polymers, in blends or composites is a strategy that can be used to attain the necessary properties and has gained much attention being the subject of several studies in the last years (Shrivastav et al., 2011).

Due to its properties, such as flexibility and thermal stability the use of PHAs with polybutylene adipate terephthalate (PBAT) has been studied as a suitable solution (Beber et al., 2018; Cunha et al., 2016; Teixeira et al., 2020). PBAT is a compostable polymer, that is biodegradable by some thermophilic organisms (Hu et al., 2010; Thumarat et al., 2012). At mesophilic temperatures, the biodegradation is much slower, and few mesophilic microorganisms have been identified thus far as PBAT biodegraders (Jia et al., 2021; Muroi et al., 2017; Palsikowski et al., 2018). Many of these new solutions are developed with great concern about the costs and function, but less attention has been given to the biodegradable issues. Even when using biodegradable polymers, a multitude of associated factors can influence and limit their biodegradation, some intrinsic to the nature of the plastics, to their physical and chemical properties, and others related to environmental abiotic factors and the microbial community present.

Driven by the knowledge resulting from the biodegrading assessment of new plastic solutions, the working plan of this dissertation was developed to evaluate the biodegradation potential of PHA/PBAT bilayer films on soil. Furthermore, the investigation provided significant insights into the understanding of the biodegradation kinetics, while also exploring the influence of abiotic factors and the ability of some microorganisms' to degrade the polymers. This can be useful for the films risk assessment, life cycle assessment and rational decision-making considering its application. More details will be given in the next chapter about the characteristics of the different types of plastics, and the main environmental factors that affect the biodegradation of plastics, with a special focus on the soil environment.

1.2 RESEARCH AIMS

The main objective of this thesis was to study the biodegradation PHA-based packaging materials in soil. The PHA-based packaging materials tested were PHAs/PBAT bilayer films produced from food by-products' substrates (i.e., cheese whey and almond shells). These objectives can be further addressed in the following specific aims:

1 - Evaluate and identify if there are physicochemical changes in the films before and after biodegradation experiments;

2 - Identify the most active/most abundant microorganisms after incubation and investigate their connection to biodegradation;

3 - Determine the importance of several variables in the process of biodegradation, namely temperature, soil moisture holding capacity and the addition of nitrogen;

4 - Identify and isolate novel PHA/PBAT degraders from soil microbiomes;

5 - Evaluate the PBAT and PHB/PBAT biodegradation using monocultures vs cocultures of the best microorganism species identified.

1.3 THESIS OUTLINE

The thesis was structured in 7 chapters. Besides the present Chapter 1, Chapter 2 provides a general introduction to key subjects addressing the biodegradation of polymers in soil, more specifically PHAs and PBAT. In Chapter 3, the biodegradation in soil of a PHBV/PBAT was analysed through the measurement of carbon dioxide (CO_2) production and focusing on the physicochemical properties of the film and especially on the isolation and identification of the microorganisms capable of degrading these polymers. In Chapter 4, the same type of approach and methodology used in the previous chapter was applied to analyse biodegradation in soil of PHB/PBAT films. In Chapter 5 the effect of soil temperature, soil water holding capacity and carbon:nitrogen ratio (nitrogen added to the soil in relation to the carbon existing in the plastic) on the biodegradation of a PHB/PBAT film was evaluated. In Chapter 6 The degradation capability of PBAT and PHB/PBAT films of 2 microorganisms identified in Chapter 4 was tested using monocultures and cocultures. In the final chapter (Chapter 7) the general conclusions regarding this dissertation are presented, as well as proposals for future works.

1.4 REFERENCES

Ashby, R., & Solaiman, D. (2008). Poly(hydroxyalkanoate) Biosynthesis from Crude Alaskan Pollock (Theragra chalcogramma) Oil. *Journal of Polymers and the Environment*, 16(4), 221–229. Retrieved from https://doi.org/10.1007/s10924-008-0108-5

Beber, V. C., de Barros, S., Banea, M. D., Brede, M., de Carvalho, L. H., Hoffmann, R., ... Wellen, R. M. R. (2018). Effect of Babassu natural filler on PBAT/PHB biodegradable blends: An investigation of thermal, mechanical, and morphological behavior. *Materials*, 11(5). Retrieved from https://doi.org/10.3390/ma11050820

Boyandin, A. N., Prudnikova, S. V., Filipenko, M. L., Khrapov, E. A., Vasil'ev, A. D., & Volova, T. G. (2012). Biodegradation of polyhydroxyalkanoates by soil microbial communities of different structures and detection of PHA degrading microorganisms. *Applied Biochemistry and Microbiology*, 48(1), 28–36. Retrieved from https://doi.org/10.1134/S0003683812010024

Cruz, M. V, Freitas, F., Paiva, A., Mano, F., Dionísio, M., Ramos, A. M., & Reis, M. A. M. (2016). Valorization of fatty acids-containing wastes and byproducts into short- and medium-chain length polyhydroxyalkanoates. *New Biotechnology*, 33(1), 206–215. Retrieved from https://doi.org/https://doi.org/10.1016/j.nbt.2015.05.005

Cunha, M., Fernandes, B., Covas, J. A., Vicente, A. A., & Hilliou, L. (2016). Film blowing of PHBV blends and PHBV-based multilayers for the production of biodegradable packages. *Journal of Applied Polymer Science*, 133(2). Retrieved from https://doi.org/10.1002/app.42165

Flury, M., & Narayan, R. (2021). Biodegradable plastic as an integral part of the solution to plastic waste pollution of the environment. *Current Opinion in Green and Sustainable Chemistry*, 30, 100490. Retrieved from https://doi.org/https://doi.org/10.1016/j.cogsc.2021.100490

Gómez, E. F., & Michel, F. C. (2013). Biodegradability of conventional and bio-based plastics and natural fiber composites during composting, anaerobic digestion and long-term soil incubation. *Polymer Degradation and Stability*, 98(12), 2583–2591. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2013.09.018

Hu, X., Thumarat, U., Zhang, X., Tang, M., & Kawai, F. (2010). Diversity of polyester-degrading bacteria in compost and molecular analysis of a thermoactive esterase from Thermobifida alba AHK119. *Applied Microbiology and Biotechnology*, 87(2), 771–779. Retrieved from https://doi.org/10.1007/s00253-010-2555-x

Jia, H., Zhang, M., Weng, Y., Zhao, Y., Li, C., & Kanwal, A. (2021). Degradation of poly(butylene adipate-co-terephthalate) by Stenotrophomonas sp. YCJ1 isolated from farmland soil. *Journal of Environmental Sciences (China)*, 103, 50–58. Retrieved from https://doi.org/10.1016/j.jes.2020.10.001

Muroi, F., Tachibana, Y., Soulenthone, P., Yamamoto, K., Mizuno, T., Sakurai, T., … Kasuya, K. (2017). Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the aerobic mesophilic bacterium Bacillus pumilus. *Polymer Degradation and Stability*, 137, 11–22. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.006

Nishida, H., & Tokiwa, Y. (1993). Distribution of poly(β-hydroxybutyrate) and poly(ε-caprolactone)aerobic degrading microorganisms in different environments. *Journal of Environmental Polymer Degradation*, 1(3), 227–233. Retrieved from https://doi.org/10.1007/BF01458031

Palsikowski, P. A., Kuchnier, C. N., Pinheiro, I. F., & Morales, A. R. (2018). Biodegradation in Soil of PLA/PBAT Blends Compatibilized with Chain Extender. *Journal of Polymers and the Environment*, 26(1), 330–341. Retrieved from https://doi.org/10.1007/s10924-017-0951-3

Pathak, S., Sneha, C. L. R., & Mathew, B. (2014). Bioplastics: Its Timeline Based Scenario & Challenges. *Journal of Polymer and Biopolymer Chemistry,* 2, 84–90.

Sang, B. I., Hori, K., Tanji, Y., & Unno, H. (2002). Fungal contribution to in situ biodegradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) film in soil. *Applied Microbiology and Biotechnology*, 58(2), 241–247. Retrieved from https://doi.org/10.1007/s00253-001-0884-5

Shrivastav, A., Mishra, S. K., Pancha, I., Jain, D., Bhattacharya, S., Patel, S., & Mishra, S. (2011). Biodegradability studies of polyhydroxyalkanoate (PHA) film produced by a marine bacteria using Jatropha biodiesel byproduct as a substrate. *World Journal of Microbiology and Biotechnology*, 27(7), 1531–1541. Retrieved from https://doi.org/10.1007/s11274-010-0605-2

Teixeira, P. F., Covas, J. A., Suarez, M. J., Angulo, I., & Hilliou, L. (2020). Film Blowing of PHB-Based Systems for Home Compostable Food Packaging. *International Polymer Processing*, 35(5), 440–447. Retrieved from https://doi.org/10.3139/217.3985

Thumarat, U., Nakamura, R., Kawabata, T., Suzuki, H., & Kawai, F. (2012). Biochemical and genetic analysis of a cutinase-type polyesterase from a thermophilic Thermobifida alba AHK119. *Applied Microbiology and Biotechnology*, 95(2), 419–430. Retrieved from https://doi.org/10.1007/s00253-

011-3781-6

Trinh Tan, F., Cooper, D. G., Marić, M., & Nicell, J. A. (2008). Biodegradation of a synthetic co-polyester by aerobic mesophilic microorganisms. *Polymer Degradation and Stability*, 93(8), 1479–1485. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2008.05.005

CHAPTER 2.
LITERATURE REVIEW

Part of the contents presented in this chapter were published on:

Fernandes, M., Salvador, A., Alves, M. M., & Vicente, A. A. (2020). Factors affecting polyhydroxyalkanoates biodegradation in soil. Polymer Degradation and Stability, 182, 109408. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2020.109408

2.1 Introduction

Plastics are synthetic and semi-synthetic polymeric compounds that are produced mainly from fossil carbon sources such as crude oil and natural gas. The major market sector in which plastics are used is packaging, being the packages mostly conceived for immediate disposal (Jain and Tiwari, 2015). In 2015, almost 96.6 % of the 146 million tons of plastic packaging placed on the market were discarded (Jain and Tiwari, 2015). Concerning packaging, only a small portion of the waste generated, about 29.5 million tons (collected plastic post-consumer waste: 34.6 % recycling, 42 % energy recovery, 23.4 % landfilling), is recovered, or recycled in Europe (Plastics Europe, 2021). Recycling is a very laborious process, that involves the collection from consumers and manual sorting. The plasticizers, additives, and colouring elements, present in plastics, significantly increase the difficulty of plastic recycling and reuse (Roosen et al., 2020). In the case of food packaging, the recycling rate is much lower due to contamination with organic substances, including oil and leftover foods (Navarre et al., 2022). Without an economic incentive, most plastics collected are sent to under-regulated countries and buried in landfills (Dauvergne, 2018). This represents an environmental problem as most plastics are very resistant to biological degradation. Packages are usually made of conventional plastics, such as polyethylene (PE) or polypropylene (PP), which are recalcitrant and accumulate for many years in landfills and the environment (Barnes et al., 2009), causing adverse effects on oceans, soil, wildlife and, possibly, on humans (Jambeck et al., 2015). With an annual production of 380 million tons, and considering the annual growth rate, it is estimated that the wasted plastic in landfills or natural environments will reach 12000 million tons in 2050 (Geyer et al., 2017). These effects and the excessive use of these materials led to significant challenges for waste treatments. Extensive efforts have been developed to create alternative plastic materials that can be competitive in financial terms, made of renewable feedstocks and that can, preferentially, undergo biodegradation, without causing harmful effects in the environment (Song et al., 2009). Nowadays, the existing plastics can be classified into four groups, regarding whether they are considered biodegradable, and the source of the feedstock used in their production as indicated in Figure 2.1.

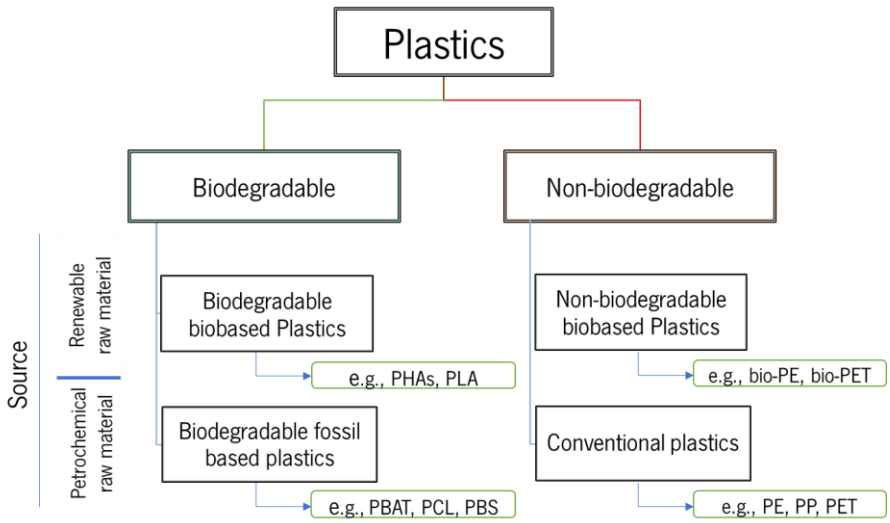

Figure 2.1 Classification of plastics according to the source of feedstock (petrochemical raw or renewable raw material) and whether they are considered biodegradable or not. Abbreviations: PHA - polyhydroxyalkanoate, PLA - polylactic acid, PBAT - polybutylene adipate terephthalate, PCL – poly(ε-caprolactone), PBS - polybutylene succinate, PET - polyethylene terephthalate, PE –polyethylene, PP – polypropylene, bio-PE - bio-based Polyethylene, bio-PET - bio-based Polyethylene terephthalate. From (Fernandes et al., 2020).

Conventional plastics, also known as oil-based or synthetic plastic, are generally derived from non-renewable resources and are generally non-biodegraded or very difficult to biodegrade (Gómez and Michel, 2013). These fossil-based plastics may also be biodegradable, and in that case, they are called synthetic biodegradable plastics. The group of bio-based plastics refers to those plastics resulting from natural resources or biomass. These plastics can be biodegradable or not and are defined as biodegradable bio-based plastics (e.g., Polylactic acid (PLA), PHAs or non-biodegradable bio-based plastics (e.g., bio-PE), respectively (Gómez and Michel, 2013). However, all the alternatives to conventional plastics combined, only represent less than 1 % of the annual global plastic production, and still 48 % of those are biobased non-biodegradable plastics (https://www.european-bioplastics.org/market/). Therefore, it is desirable, that the production capacity of those alternatives increases from the actual 2.22 million tonnes to 6.3 million tonnes in 2027 (https://www.european-bioplastics.org/market/). Table 2.1 indicates the annual production of conventional plastics alternatives as well as the main companies.

Table 2.1 Commercial bioplastics polymers. Main applications of bioplastics, with its annual production and which conventional plastics they can replace

Main Applications	Main Conventional plastic	Bioplastic alternative	Annual production (tons)*	Main Companies	Biodegradability	References
Food packaging, compostable films and bags, biomedical scaffolds, tissue engineering;	PE and others	Starch-based	397380	Novamont	Biodegradable	(Żołek-Tryznowska and Holica, 2020)
Packaging materials, medical implants, 3D printing polymers, textiles, electronics;	PET	PLA	459540	GreenStripe® Cold cups; NatureWorks; Corbion and Total; BBCA Group	Compostable	(Arrieta et al., 2013; Mathew et al., 2005)
Flexible packaging and others;	Several conventional plastics	Cellulose	79920	VTT; Stora Enso	Biodegradable	(Su et al., 2020)
Packaging;	PET	Bio-PET	93240	Virent and Coca-Cola	Bio-based non-biodegradable	(Xiao et al., 2015)
Flexible and rigid packaging, consumer goods, transport;	PP	Bio-PP	86580	Neste and Borealis	Bio-based non-biodegradable	(Adediran et al., 2022)
Packaging bags and films, automotive applications;	PE	Bio-PE	328560	Braskem “I’m Green”	Bio-based non-biodegradable	(Byun and Kim, 2014)
Cling wraps for food packaging, compostable plastic bags, mulch films;	PET	PBAT	99900	BASF; Jinhui Zhaolong High	Compostable	(Moustafa et al., 2017; Souza et al., 2019)
Packaging, compost bags, tissue engineering;	PE and others	PHA	86580	Bio-On; RWDC Industries; Danimer Scientific; Tianjin GreenBio Materials, CheilJedang	Biodegradable	(Bucci et al., 2005; Chen and Wu, 2005)

Films and sheets for food packaging and agriculture, compost bags, fishing nets, automotive industry;	PP and PET	Bio-PBS	19980	PTT MCC Biochem; BASF and Corbion; Hexing Chemical	Biodegradable	(Gualandi et al., 2012; Xu and Guo, 2010)
Packaging of soft and alcoholic beverages; fibers for textiles;	PET	PEF	–	Avantium;Corbion;SULZER	Bio-based non-biodegradable	(Loos et al., 2020)
Fiber for textiles, nonwoven fabrics;	PET	Bio-PTT	295260	Sorona®/DuPont Corterra™/Shell Chemicals	Bio-based non-biodegradable	(Rahman et al., 2023)
Automotive, electrical and electronics, consumer goods, sports and leisure;	PP	Bio-PA	246420	EcoPAXX®/DSM; Vestamid® TERRA/Evonik	Bio-based non-biodegradable	(Pervaiz et al., 2016)

Abbreviations: bio-PA - bio-based Polyamide; bio-PE - bio-based Polyethylene; PEF - Polyethylene Furanoate; bio-PET - bio-based Polyethylene Terephthalate; bio-PP - bio-based Propylene; bio-PBS - bio-based Polybutylene Succinate; PHA - Polyhydroxyalkanoate; PLA - Polylactic Acid; bio-PTT - bio-based Polytrimethylene terephthalate; PBAT - polybutylene adipate terephthalate. * - adapted from https://www.european-bioplastics.org/market/.

2.2 Definition of biodegradation

The American Society for Testing and Materials (ASTM), the International Standardization Organization (ISO) and the European Committee for Standardization (CEN) have developed standardized definitions for the terms “biodegradable plastics” and “biodegradability” (Table 2.2). The definitions are all similar, however, only the CEN definition for biodegradable plastics indicates that the material needs to be converted to water, CO_2 and/or methane and new cell biomass. The ISO and ASTM definition only indicate a significant change in chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application.

Table 2.2 Definitions used by different organizations (ASTM, CEN, ISO) related to biodegradation

Organization		Definition
CEN	Biodegradable plastics	A degradable material in which the degradation results from the action of microorganisms and ultimately the material is converted to water, CO_2 and/or methane and new cell biomass (Pagga, 1998)
	Biodegradation	Degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of a material (Pagga, 1998)
	Inherent biodegradability	The potential of a material to be biodegraded, established under laboratory conditions (Pagga, 1998)
	Ultimate biodegradability	The breakdown of an organic chemical compound by microorganisms in the presence of O_2 to CO_2, water and mineral salts of any other elements present (mineralization) and new biomass or in the absence of O_2 to CO_2, methane, mineral salts, and new biomass (Pagga, 1998)
ASTM D883-18 (2018) - Standard Terminology Relating to plastics	Degradable plastic	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification.
	Biodegradable plastics	A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi, and algae.
ISO 472 (2013) Plastics — Vocabulary	Degradable plastic	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions, resulting in the loss in some properties, as measured by standard test methods appropriate to the plastic and the application, in a given period of time that determines whether the plastic can be classified as biodegradable or not
	Biodegradable plastics	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period that determines its classification. The change in the chemical structure results from the action of naturally occurring microorganisms
	Degradation	Irreversible process leading to a significant change in the structure of a material, typically characterized by a change of properties (e.g. integrity, molecular mass or structure, mechanical strength) and/or by fragmentation, affected by environmental conditions, proceeding over a period of time and comprising one or more steps
	Biodegradation	Degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of a material

Ultimate aerobic biodegradation	Breakdown of an organic compound by microorganisms in the presence of oxygen into carbon dioxide, water, and mineral salts of any other elements present (mineralization) plus new biomass

The term 'bio-based' used commonly in the literature causes some misinterpretations. A bio-based plastic implies that the material is (partly) derived from biomass (plants). Partially bio-based, (or hybrid) plastics, are synthesized with both renewable and conventional fossil fuel-based carbon (Atiwesh et al., 2021). It is important also to clarify the term bioplastic, which is often misused and misleading. Bioplastic is a group of polymers that are bio-based, biodegradable, or both (Atiwesh et al., 2021). In this case, this definition includes all the groups presented in Figure 2.1, apart from the conventional plastics.

2.3 Types of bioplastics

2.3.1 Bio-based non-biodegradable

Several types of plastics are part of the bio-based non-biodegradable category such as polyurethane, polyvinyl chloride and polycarbonates among others. However, bio-PE, bio-PP and bio-PET stand out because their petrochemical versions are the most used worldwide (Ali et al., 2021).

PE is one of the most broadly used commodity thermoplastics, including for packaging (plastic bags, plastic films, and containers such as bottles). Although PE is mainly produced from crude oil, natural gas or methane after dimerization, it can also be produced from the fermentation of sugarcane, so the ethylene monomers are synthetized by dehydration of bio-ethanol, obtained from glucose (Demuner et al., 2019). However, bio-PE is non-biodegradable and is recyclable with the same properties as fossil-based polymers. At a commercial scale, bio-PE started to be produced by Braskem company, mainly for food packaging, cosmetics, personal care, and automotive applications (Byun and Kim, 2014).

PP is the second most used commodity plastic, and can also be made from renewably sourced feedstock, by butylene dehydration of bio-isobutanol produced from glucose and later polymerization (Kikuchi et al., 2017). PP can also be produced through methanol using the same industrial infrastructures as petrochemical methanol. However, the scale-up is not in an advanced stage as the bio-PE production due to the limited knowledge concerning some process parts.

PET is the third most common thermoplastic and can be synthetized by esterification of terephthalic acid and ethylene glycol. To produce bio-PET, renewable ethylene glycol (MEG) can be used and it is obtained from sugarcane-derived ethylene, and a bio-terephthalic acid can also be produced by different routes

including starting from sugar. It is mainly used for synthetic fibers and packaging, especially bottles (Xiao et al., 2015). The advantages of producing these polymers are, using biomass (such as by-products) coming from food and agricultural wastes, and the fact that they don't require major changes and investments in the existing waste management infrastructures. The main challenges include reducing the high production and processing costs, preventing competition with food production, and reducing agricultural soil use and forests. Nonetheless, these polymers although easier to implement since they are chemically identical to their petrochemical versions, are also not biodegradable, imposing the same environment threats and challenges.

2.3.2 Fossil-based biodegradable polymers

This category comprises polymers that are more interesting in terms of biodegradation than conventional plastics, however, they are still produced from fossil carbon sources. This origin creates an imbalance in the carbon cycle because it will be necessary millions of years to transform the biomass into the original form. The main polymers in this category include poly(ε-caprolactone) (PCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA) and poly(alkylene dicarboxylate such as polybutylene succinate (PBS).

PCL is a semi-crystalline aliphatic polyester, produced by the ring-opening of aromatic of ε-caprolactone obtained from fossil carbon sources (Chrissafis et al., 2007). Due to its rheological properties, is relatively simple to process, so it can be used for packaging purposes, being also thermoplastic, and resistant to water and oil, it can used as compostable bags and for food packaging (Martínez-Abad et al., 2013). It can clearly be biodegraded by microorganisms. PCL-degraders are relatively common in the environment and use mostly lipases and esterases (Nawaz et al., 2015). The low melting temperature of about 60 °C, prevents its use in various applications (Chrissafis et al., 2007). The use of composites or blends may be a solution to surpass the PCL disadvantages (Chrissafis et al., 2007; Mofokeng and Luyt, 2015).

A plastic that can be produced by various chemical routes is PGA. The easiest of which is the direct poly-condensation polymerization of glycolic acid. It can also be synthesized by solid-state polycondensation of halogen acetates or by reacting formaldehyde (trioxane) with carbon monoxide (CO). However, the ring-opening polymerization of glycolide, the cyclic dimer of glycolic acid, is the industrial method commonly used (Ayyoob et al., 2017; Gautier et al., 2009; Göktürk et al., 2015; Takahashi et al., 2000). It is a tough fiber polymer, cheap, with high mechanical strength, toughness, melting point (219-227 °C), crystallinity and excellent barrier properties (Gautier et al., 2009). The advantage is that can be easily extruded and moulded with other polymers making it useful for several packaging and industrial applications. Even

though it has exceptional properties, the production is low because of limitations in monomer production (glycolide or glycolic acid) allied to the high cost.

PVA is created from polyvinyl acetate monomer by the polymerization and partial hydrolysis of ethanol with potassium hydroxide, and in water it is a solubilized crystalline structure polymer (Razzak et al., 2001). PVA is a biodegradable polymer and the hydrolysis of the hydroxyl groups on the carbon atoms increases the biodegradability (Corti et al., 2002)**.** PVA presents superior characteristics as good oxygen barrier, nonetheless, to avoid the degradation of its permeability toward gas, it must be protected from moisture (Limpan et al., 2012). PVA needs to be cross-linked to form hydrogels for use in several applications. PVA is used in biomedical applications since it is biocompatible and is used in cartilage and orthopedic applications among others, and is also used in the paper industry, and food packaging industry (Bispo et al., 2010; Tripathi et al., 2009).

PBS is an aliphatic polyester produced from the polycondensation reaction of the aliphatic dicarboxylic acid (succinic acid) and 1,4-butanediol (Jiang et al., 2018). These monomers are mostly obtained from fossil-based resources but can also be produced from renewable resources. Succinic acid can be derived from bacterial fermentation of sustainable feedstock, such as wheat milling by-products (Dorado et al., 2009). 1,4-butanediol can be obtained from renewable feedstock fermented through a process that produces succinic acid that is then purified and reduced catalytically (Forte et al., 2016). It has some interesting physical properties including a semicrystalline nature, a lower melting point, thermal stability, good gas barrier properties, and decent processing properties (Qiu et al., 2003; Wang et al., 2012). Nonetheless, it has poor mechanical properties, namely poor impact strength, which limits its use in many applications (Huang et al., 2009). It is considered a biodegradable polymer and even mesophilic bacteria are effective in its biodegradation (Tezuka et al., 2004). It is mostly used in the biomedical area such as drug delivery applications, but also in fishing nets, forestry, or civil engineering (Gualandi et al., 2012; Xu and Guo, 2010).

2.3.2.1. PBAT

PBAT is a polymer created by a polycondensation reaction of butanediol, adipic acid and terephthalic acid, with common polyester production technology and equipment. The chemical structures of the different monomers are shown in Figure 2.2. As polycondensation catalysts, tin, titanium, and zinc could be employed (Witt et al., 1995). The process of production consists of pre-mixing, pre-polymerization and final polymerization. To eliminate water, and to improve the condensation reaction, high temperatures and vacuum with extended reaction times are necessary (Witt et al., 1995). To improve the crystallization

behaviour and prevent sticking, inorganic compounds are used as nucleating agents such as talc, chalk, mica, or silicon oxides in the final polymerization step. It is mainly produced from fossil carbon sources, although some efforts are being made to produce all of the constituents from bio-based sources (Kruyer and Peralta-Yahya, 2017; Tachibana et al., 2015; Yim et al., 2011).

Due to the aromatic unit in the molecule chain, PBAT presents some interesting mechanical properties, being more flexible than other polyesters, including PLA and PBS, and has analogous mechanical properties to the low-density PE (LDPE) (Yamamoto et al., 2005). Some of the properties include a melting point of 115 to 125 °C, crystallinity point of 60 °C, tensile strength of 21 MPa, elongation at break 670 %, a flexural strength of 7.5 MPa and a melt flow index at 190 °C under 2.16 kg around 4, making it very appropriate for blowing film application (Jian et al., 2020). Since PBAT has 2 different units, and the mechanical properties are influenced by monomer composition and molecular weight, it can be tailored.

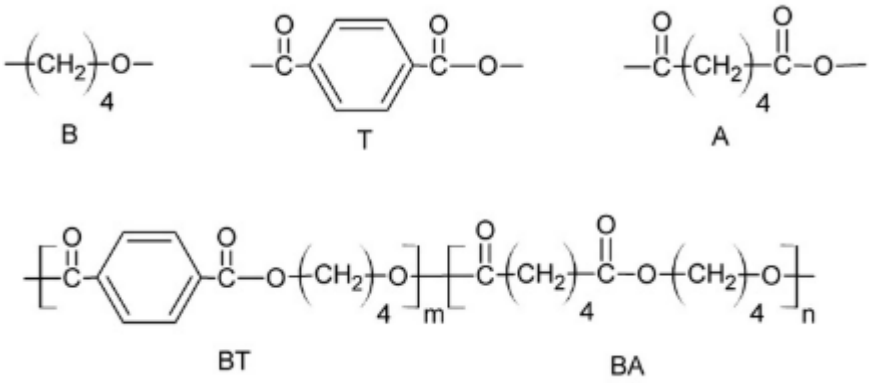

Figure 2.2 Structure of 1,4 butanediol (B), terephthalic acid (T), adipic acid (A), and PBAT (BT – butylene terephthalate and BA - butylene adipate) (Kijchavengkul et al., 2010a).

Packaging, particularly food packaging, is the main sector where PBAT is used (Moustafa et al., 2017). Several commercially compostable PBAT-based materials are available and can be used to make packages, being some of the main companies BASF and Novamont. It is also used for agriculture purposes as mulch films (Souza et al., 2019). Mulch films, are used to increase crop yields because they can increase soil temperatures, maintain soil moisture, control weed development and protect against adverse weather and pests. Conventional plastics are mostly used, such as PE but they are difficult to recover due to embrittlement and fragmentation, leading to their accumulation in the soil (Souza et al., 2019). Nonetheless, it has a high manufacturing cost, and poor mechanical and thermal properties when compared to conventional nonbiodegradable polymers, which reduces its commercial applications in some areas (Moustafa et al., 2017). This problem has been minimized by blending PBAT with other more

cost-effective biodegradable polymers or adding fillers, compatibilizers, and plasticizers to create cost-effective composites to increase the applications and enhance the properties.

2.3.3 Bio-based biodegradable polymers

The polymers in this category have received massive attention as viable replacements to petroleum-based polymers for a large range of industrial, agricultural and health applications. They are produced from natural origins (plants, animals or microorganisms) such as lipids (e.g., plant oils and animal fats), polysaccharides (e.g., alginate, cellulose, chitin and chitosan, lignin and starch) and proteins (e.g., albumin, casein, gelatine, silk, wheat gluten and whey). Other polyesters produced by microorganisms (e.g., PHAs) or synthesized from bio-derived monomers (e.g., PLA) are also in this category. Some of them present several advantages, including the possibility of being produced from byproducts of other industrial processes (Pietrini et al., 2007). Biodegradable plastics are interesting for plastic waste management since their decomposition delivers rich metabolites to soil and avoids the accumulation of waste in landfills, while reducing the cost of waste management and the emission of greenhouse gases (Bher et al., 2022).

Chitosan is a linear polysaccharide consisting of randomly distributed. β (1-4) polymer of N-acetyl-D-glucosamine and *N*-acetyl *D*-glucosamine that exist in several marine invertebrates including lobsters, crabs, and shrimps, and in insects, fungi, and yeasts (Hu et al., 2007). It is produced from chitin by the removal of acetyl groups when crustacean shells are treated with an alkaline substance. Is broadly applied in the medical department, specifically for making artificial skin, sutures, cosmetics, wound treatment, and drug carriers (Gomaa et al., 2010; Madhumathi et al., 2009, 2010; Mutalik et al., 2008). They have no antigenic properties and are insoluble in water (hydrophobic in nature) because of intermolecular hydrogen bonds. This polymer presents unsatisfactory mechanical properties (including insolubility) and is often used in combination with other polymers and additives (Mitsumata et al., 2003).

A protein generally obtained from animal parts such as skin and bones but recently fish and poultry have also been used, is collagen (Enescu et al., 2015). Gelatin is a combination of peptides and protein that is produced from collagen through hydrolysis in acidic or alkaline conditions (Zhuang et al., 2015). Gelatin has gel-forming properties that offer many applications, especially in the food industry, such as edible and biodegradable films and coatings (Nur Hanani et al., 2014). Gelatin films have good biological properties, but they vary depending on the film's formulation and source. However, the relatively weak thermal stability, poor mechanical properties, and sensitivity to moisture (poor barrier properties against

water vapor) decrease its potential application (Kozlov and Burdygina, 1983). This can be improved using combinations with other biopolymers (Limpisophon et al., 2010).

The most used biodegradable aliphatic polyester is PLA, which results from the fermentation of glucose, which is extracted from sources such as sugar, potatoes, or sugarcane (Mathew et al., 2005). PLA is a lactic acid cyclic dimer produced from lactide ring-opening polymerization or D- or L-lactic acid polycondensation (Conn et al., 1995). Is the first polymer created on an industrial level from renewable resources, and is easy to process (Rudnik and Briassoulis, 2011a). It has great mechanical properties comparable to PET and PP, namely good stiffness, and strength (Mathew et al., 2005). It is mainly used as plastic bags for domestic waste, food packaging, and domestic utensils such as disposable cups and plates (Arrieta et al., 2013; Mathew et al., 2005). PLA products are considered compostable (Husárová et al., 2014). Nonetheless, biodegradation in natural environments such as soils is quite slow (Rudnik and Briassoulis, 2011a). Besides this question the major PLA drawbacks in terms of properties are the low toughness and thermal stability, so the incorporation of other materials can be a solution to improve these characteristics (Mathew et al., 2005).

Pectin is a naturally occurring polysaccharide extracted from citrus peels and apple pomace that have 10-15 % or 20-30 % of pectin respectively, under certain acidic conditions, and is present in the plant middle lamella. It is composed of D-galacturonic acid units linked by (1-4) glycosidic linkages (Mukhiddinov et al., 2000). It is a water-soluble biopolymer and a traditional gelling agent used to make jams and jellies, ionotropic gelation, and gel coating (Mukhiddinov et al., 2000). It has also several biochemical applications including drug delivery, gene delivery tissue engineering and wound healing (Katav et al., 2008; Munarin et al., 2011; Oechslein et al., 1996).

Lignin is composed mainly of phenolic alcohols such as monolignols, (e.g., ρ-coumaric alcohol) and is extracted from wood, corn stalks, wheat and rice straw and flax (Buranov and Mazza, 2008). Lignin has been widely studied in the material science field because of its abundance, biodegradability, low cost, biocompatibility, and antioxidant properties (Pouteau et al., 2003). Lignin is more resistant to chemical and biological attacks than cellulose (Pouteau et al., 2003). It can be used to obtain biofuels through pyrolysis, followed by catalytic upgrading of the resulting materials. A fast pyrolysis is used to obtain oil and a slow one to obtain char (Bai and Kim, 2016). It can be used in the agriculture field in the development of nanocarrier systems for the controlled release for example of pesticides (Beckers et al., 2020). It can also be used as a sizing agent in the paper industry, as a dispersant and as a binder (Aso et al., 2013; Jiang et al., 2020; Tanase-Opedal and Ruwoldt, 2022).

Alginate is a natural polymer consisting of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, that can be mostly found in brown algae (i.e., *Durvillaea, Laminaria, Lessonia, Macrocystis, Sargassum*), as a calcium, magnesium, or sodium salts (Günter et al., 2020). Calcium alginates are insoluble in water and have been used in the food industry to coat fruits and vegetables, as alginate-based films for water-soluble powder products such as coffee and powdered milk, and in the pharmaceutical industry for products that require solubilization in hot water such as vitamins (Gundewadi et al., 2018; Puscaselu et al., 2019). It has been also used in the biomedical area due to its biocompatibility, biodegradability, superior ability to incorporate and release proteins, cell affinity, effective bio adhesion and absorption characteristics for wound dressing and drug delivery agents among others (Shin et al., 2020; Witzler et al., 2021).

Starches are formed by large glucose units linked by glycosidic bonds. They are found in several plants such as wheat, corn, rice, and potatoes. they are normally used in granules and are formed by one branched (amylopectin, 80 %) and one linear polymer (amylose 20 %) (Sanyang et al., 2018). They have several applications in the fields of drug delivery, tissue engineering, biomedical scaffolds, and food packaging (Żołek-Tryznowska and Holica, 2020). Starch is non-toxic, abundant, biocompatible, edible, has low cost, and is biodegradable. Nonetheless, it has some weaknesses when used in a food packaging context, such as water susceptibility, brittle mechanical behaviour, poor barrier properties, and some resistance to extreme processing conditions (Baghi et al., 2022).

Cellulose, the most abundant biopolymer on earth, is a polysaccharide consisting of a linear chain of several hundred to many thousands of β-D-glucopyranose units, linked by (1–4)-glycosidic bonds (Su et al., 2020). It can be found in wood, agricultural residues, factory and food wastes, and some types of grass, along with microbial biosynthesis (algae, fungi, bacteria) (Cheng et al., 2009; Liu et al., 2020a; Zhao et al., 2019). Bacterial cellulose doesn't require the removal of impurities (Cheng et al., 2009). They have interesting properties such as being edible, biodegradable, lightweight, nontoxic, bioavailable and they are also cheap (Su et al., 2020). Cellulose is especially known for its use in paper, textile, and pulp production units. Bacterial cellulose has different properties, and it has been studied in the food industry as a thickening or gelling agent, stabilizer, water-binding additive, and also as food packaging material. Although with high thermal resistance and good physical and mechanical properties, the high-water absorption capacity and insufficient interfacial adhesion, restrict its use (Baghi et al., 2022). In biodegradation tests, cellulose is commonly used as a positive control.

One of the most interesting type of polymers in this group are the PHAs. The PHA family of polymers is considered biodegradable, non-toxic, environmentally friendly, and can be produced from renewable

resources (El-Abd et al., 2017; Wang et al., 2017). Nowadays, PHA polymers have the potential to compete with conventional plastics due to their characteristics which can include a high degree of polymerization crystallinity and insolubility in water (El-Abd et al., 2017; Wang et al., 2017).

2.3.3.1. PHAs

PHAs are biopolyesters that accumulate intracellularly in a wide range of microorganisms, such as bacterial and archaeal cells, through the fermentation of sugars and lipids, and they function mainly as energy and carbon storage compounds (Cruz et al., 2016). These polyesters are produced when bacterial growth is restricted by depletion of nitrogen or phosphorous, and when an excess of carbon source is available (Shang et al., 2003). PHAs guarantee the survival of bacteria under limited nutrient conditions by acting as carbon and energy reserves. PHAs are composed of 3-hydroxy fatty acid monomers, which form linear, head-to-tail polyester. They are typically polymers of 103 to 104 monomers, which accumulate as inclusions of 0.2-0.5 μm in diameter (Chaudhry et al., 2011). These inclusions or granules are synthesized and stored by both gram-positive and gram-negative bacteria without harmful effects on the hosts. Depending on the number of carbon atoms in their monomeric constituents (3-hydroxyalkanoate units), PHAs can be classified as short-chain-length (scl-PHA), which consists of monomers of 3–5 carbon atoms, and medium-chain-length (mcl-PHA), in which the monomers contain more than 6 carbon atoms (Ashby and Solaiman, 2008). The scl-PHAs are considered thermoplastic due to their relatively high crystallinity and their properties that resemble those of some petrochemical-based polymers. Whereas mcl-PHAs present minimal crystallinity and exhibit elastomeric and/or free-flowing properties (Ashby and Solaiman, 2008).

The existence of PHAs in bacteria has been known since 1926 when Lemoigne reported the formation of polyhydroxybutyrate (PHB) inside bacteria *(Bacillus megaterium*) (Liu et al., 2019). Microbial biosynthesis of PHB (Figure 2.3) initially starts with 3-ketothiolase (PhaA) that combines two molecules of acetyl-CoA originating acetoacethyl-CoA. Acetoacethyl-CoA reductase (PhaB) catalyses the reduction of acetoacetyl-CoA by NADH to 3-hydroxybutyryl-CoA. Then PHB synthase (PhaC) uses hydroxybutyryl-CoA as a monomer and polymerizes 3-hydroxybutyryl-CoA to PHB, releasing coenzyme-A (Tsuge et al., 2005).

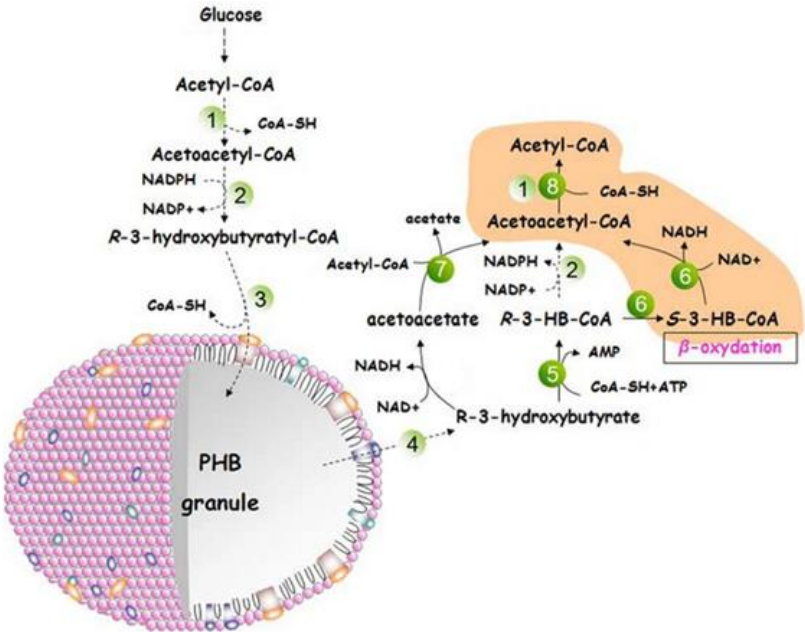

Figure 2.3 Metabolic network between PHB biosynthesis and mobilization in *Escheria. coli.* ① β-ketothiolase, PhaA ② NADPH-dependent acetoacetyl-CoA reductase, PhaB ③ PHB synthase, PhaC ④ PHB depolymerase, PhaZ ⑤ acyl-CoA synthetase or thioesterase ⑥ 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxybutyryl-CoA epimerase, FadB ⑦ acetoacetyl-CoA transferase, AtoA, AtoD ⑧ acetyl-CoA acetyltransferase, AtoB. Adapted from (Wang et al., 2009).

Through normal bacterial growth, the 3-ketothiolase is repressed by free coenzyme-A, which comes out of the Krebs cycle. When the entry of acetyl-CoA in the Krebs cycle is limited, (e.g., nutrient limitation), the surplus acetyl-CoA is directed into PHB biosynthesis (Colin and BjØrn, 2006). If the bacteria use different substrates, copolymers such as polyhydroxybutyrate-co-hydroxyvalerate (PHBV) can be produced (Pederson et al., 2006). This occurs because the substrates are metabolized by different metabolic pathways (Verlinden et al., 2007).

PHAs can also be synthetically produced, being the most interesting techniques the retrosynthesis and ring-opening reactions. In the first process, propylene oxide (acquired from propylene from crude oil) is combined with CO, obtained from petroleum gas, fuel oil, or biomass (Winnacker, 2019). Other alternatives include the production of PHB by ring-opening reactions of cyclic compounds such as racemic b-butyrolactone. The polymeric polymer chain is formed through consecutive additions of the open structures of racemic b-butyrolactone using metal initiators (Ajellal et al., 2009). Other components may be also used such as racemic cyclic diode derived from biological succinate to obtain PHB through ring opening (Tang and Chen, 2018). In this case, the PHB obtained has analogous isotacticity to biological PHB (high melting temperature and molecular weight). Nevertheless, the catalysts used may have metals

such as chromium, a heavy metal that can be toxic (Vagin et al., 2015). It is necessary to remove these compounds before general use. It should also be considered the use of bio-sourced materials because even if economically viable, the use of petroleum-based components to synthetically produce PHAs considerably diminishes some of its advantages.

PHA bioaccumulation is common in domains of Bacteria and Archaea with PHA-producing organisms belonging to more than 70 genera (Lu et al., 2009; Poli et al., 2011). Most species of bacteria that produce PHA are Gram-negative from genera *Azohydromonas*, *Burkholderia*, *Pseudomonas*, and *Cupriavidus* (Lu et al., 2009). *Cupriavidus necator (*previously *Wautersia eutropha)* is the species that has been most widely studied (López-Cuellar et al., 2011). PHA production in Gram-positive bacteria has been described in genera *Bacillus, Caryophanon, Clostridium, Corynebacterium, Micrococcus, Microlunatus, Microcystis, Nocardia, Rhodococcus, Staphylococcus, Streptomyces* (Lu et al., 2009). PHA is also found in archaea but limited to *haloarchaea* species, specifically of the genera *Haloferax, Halalkalicoccus, Haloarcula, Halobacterium, Halobiforma, Halococcus, Halopiger, Haloquadratum, Halorhabdus, Halorubrum, Halostagnicola, Haloterrigena, Natrialba, Natrinema, Natronobacterium, Natronococcus, Natronomonas*, and *Natronorubrum* (Han et al., 2010). Bacteria are capable of accumulating about 30 to 80 % of their dry weight in the form of intracellular granules as carbon and energy reserves (Madison and Huisman, 1999). Microalgae and/or cyanobacteria also have species capable of producing and accumulate PHAs (Bhati and Mallick, 2012; Nishioka et al., 2001).

Around 150 different PHA monomers have been identified (Steinbüchel and Lütke-Eversloh, 2003). This number can continually grow due to the creation of new kinds of PHA that are produced with chemical or physical modification of naturally occurring PHA (Zinn and Hany, 2005). The production of PHA can occur through several carbon sources. They include gases such as methane, n-alcohols such as ethanol, n-alkanes such as octanes, n-alkanoic acids such as oleic acid, and saccharides such as fructose or glucose (Conte et al., 2006; Santhanam and Sasidharan, 2010; Yamane et al., 1996). Other sources have also been reported, namely waste streams that comprise for example, plant oil mill effluents, frying oil waste, vinegar waste, waste fats, food waste, and agricultural waste (Koller et al., 2009). Because of the high price of production, due to factors such as carbon source and nutrients, exhaustive research has been conducted to expand PHA production from lower-cost carbon sources and waste carbon as a means to lower the cost of production (Morais et al., 2014). In this sense, the co-culturing of microbial strains has been useful, allowing the use of alternative types of carbon substrates. In these cases, the first microorganism to transform the carbon substrate into a metabolite that can be later consumed by a

second microorganism for PHA production. For example, *Cupriavidus necator* cannot efficiently metabolize sugars, whey or starchy waste, but when cultivated together with lactic acid-producing bacteria these substrates can be transformed into lactate, that can be then used by *C. necator* to produce PHAs (Tanaka et al., 1995).

Several methods have been used for the detection and analysis of intracellular PHA. These methods can be used in the identification of new PHA-producing organisms or for the monitoring of the production of PHA. Polymerase chain reaction (PCR) can be used for gene detection. So, primers can be designed to amplify specific genes such as those responsible for enzymes that intervene in PHA production (Romo et al., 2007). Cell staining with Nile red or Nile blue dye allows, under UV illumination, the detection and identification of organisms that produce PHA (Spiekermann et al., 1999). Transmission electron microscopy (TEM) allows direct visualization of PHA, by presenting intracellular granules which indicates its bioaccumulation (Bassas Galià, 2010). For quantification, the crotonic acid assay was commonly used with PHB, however, this method can overestimate the amount of PHB (Law and Slepecky 1961). Nowadays, other techniques are used, such as Fourier transform infrared spectroscopy (FTIR), that can detect diverse types of PHA (Hong et al., 1999). Liquid chromatography (LC) and gas chromatography (GC) are the techniques more commonly used because they can offer information about the composition of PHA and give precise quantification. GC is the preferred method for PHA qualitative and quantitative analysis due to its sensitivity and high separation capacity (Rijk et al., 2005).

To extract the intracellular PHA granules produced by the bacteria, it is necessary to use wall/cell membrane lysis. Initially, the bacterial cells that contain PHAs are separated from the medium by centrifugation. Organic solvents such as acetone, chloroform, methylene chloride, or dichloroethane are the methods most used to recover intracellular PHA (Jiang et al., 2006b; Ramsay et al., 1994). Alternatives to organic solvents are digestion methods, with sodium hypochlorite (Berger et al., 1989) or enzymatic digestion procedures using for example, sodium dodecyl sulphate (SDS) (Kapritchkoff et al., 2006; de Koning et al., 1997). Other alternatives, for example, a new cultivation method that enables spontaneous discharge of PHB stored inside *Escherichia coli* up to 80 % has been proposed (Jung et al., 2005).PHB is the most common and studied polymer within the PHA family (Volova et al., 2017). Other polymers that are part of the PHA class are poly(4-hydroxybutyrate) (P4HB), poly(3-hydroxyvalerate)

(PHV), poly(3-hydroxyhexanoate) (PHH), poly(3-hydroxyoctanoate) (PHO) and their copolymers, some of them represented in Figure 2.4

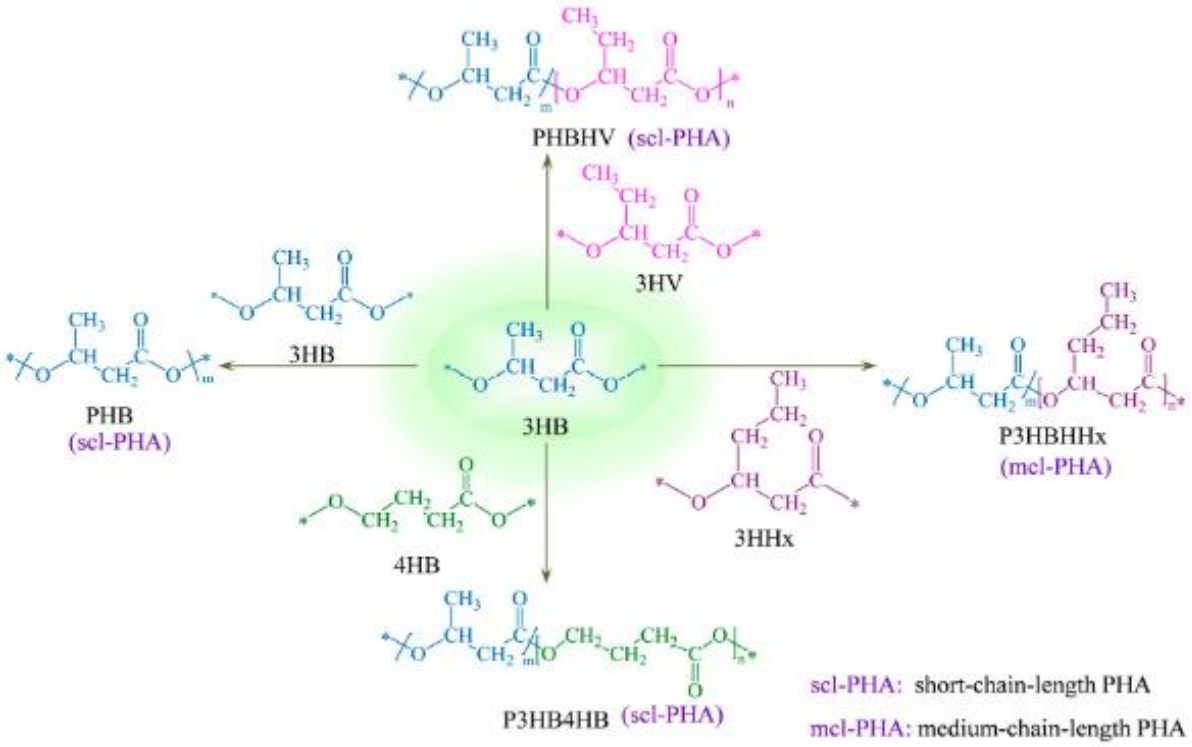

Figure 2.4 Representation of the structure of several copolymers of PHA (Wang et al., 2016).

PHA polymers have different monomer composition, physicochemical properties, size, and structure, because they can be produced by different microorganisms and with different substrates (Ha and Cho, 2002). Some of these properties, including lower environmental impact, make them ideal as replacements for petrochemical polymers presently used for packaging and coating applications (Pietrini et al., 2007). The main applications are for packaging such as containers and films (Bucci et al., 2005). In the medical field, PHAs also have many applications. PHB is even compatible with the blood and tissues of mammals and can be used as repair patches, orthopaedic pins, adhesion barriers, stents, nerve guides, and bone marrow scaffolds (Chen and Wu, 2005).

2.4 Microbiology of PHA and PBAT biodegradation

2.4.1 PHAs biodegradation in soil

In the last decades, soil has become one of the environments most heavily polluted due to the disposal of several types of waste, including hazardous waste, organic pollutants, and non-degradable plastics (EC, 2006). Nevertheless, it has a vast diversity of microorganisms, which increases the possibility of finding

plastic-degrading microbes. The soil pollution problem caused to a higher extent by plastic waste can be somewhat alleviated by the manufacture and use of advanced bio-based biodegradable plastics (Briassoulis and Mistriotis, 2018). From the category bio-based plastics, the PHA family shows more potential to be implemented as the best alternative to conventional plastics, because PLA (the other well-studied biodegradable and bio-based plastic material) is relatively resistant to biodegradation by soil microorganisms since soil bacteria were reported to produce a scarce amount of PLA degrading enzymes (Ohkita and Lee, 2006). Nevertheless, PLA is quickly degraded in soil or compost at high temperatures (> 45 °C) (Karamanlioglu and Robson, 2013). It is estimated that PHB biodegraders are widespread in different environments in percentages ranging from 0.2 to 11.4 % of the total microflora, such as soils, sewage sludge, landfill and pond sand, which is a huge advantage (Nishida and Tokiwa, 1993a). Soil is also considered an environment with an excellent capacity for degrading PHA (Boyandin et al., 2012a). It has been estimated that the percentage of bacteria capable of degrading scl-PHA in the soil is between 2 to 18 % of the total colonies formed from soil plating (Suyama et al., 1998).

Table 2.3 summarizes the laboratory biodegradation tests conducted with PHA polymers. Gómez and Michel, (2013) tested the biodegradability of several commercially available alternative materials for conventional plastic, according to the ASTM D5988-03. During incubation in soil for 660 days (Table 2.3), CO_2 production was monitored, and the results showed that the maximum biodegradability was obtained for PHA films (around 70 %), which was not statistically different from that of cellulose paper (control). The scanning electron microscopy (SEM) analysis presented considerable disintegration of PHA-based plastic compared to the other materials tested (Gómez and Michel, 2013). In natural soils, polymers from the PHA family present higher biodegradation rates (evaluated by weight loss) when compared to other polymers such as poly-DL-lactide and ethyl cellulose (Woolnough et al., 2010). These results may be due to higher biofilm development on PHA plastic films (Woolnough et al., 2010).

Table 2.3 Laboratory soil biodegradation tests for PHA polymers, with information about the type of soil conditions, biodegradation reached and length

Type of Plastic	Type of material	Type of environment	Conditions	Test method	Biodegradation (%)	Length of test (days)	References
PHB PHB/eucalyptol (3 %) PHB/thymol (3 %) PHB/limonene (3 %)	Films	Soil	room temperature	ISO 17556	100 66.4 77 73.3	<200 226 226 226	(Fayyazbakhsh et al., 2022)

PHB PHBV PHB/agave fiber (80 %/20 %) PHBV/agave fiber (80 %/20 %) PHB/agave fiber propionylation (80 %/20 %) PHBV/agave fibre propionylation (80 %/20 %)	Films	Soil/compost/chicken manure/ sheep manure (60/20/10/10 %)	38 % humidity, pH 8.5–8.8	ISO 20200, ISO 14855	34 40 41 58 45 47	32 31 31 33 36 32	(Gallardo-Cervantes et al., 2021)
PHB PHA	Films	commercial soil	23 °C, 33 % moisture	ASTM D5988-03 (weight loss)	82,24 76.30	80	(Pérez-Arauz et al., 2019)
PHA-g-MA/silane treated palm fiber (80/20 %) PHA-g-MA/silane treated palm fiber (60/40 %) PHA/marine algae powder (90/10 %) PHA/marine algae powder (80/20 %)	Powder	greenhouse-room soil-planting	25 °C, 35 % moisture	weight loss	61 78 70 88	120	(Wu, 2016)
PHB	Films	Garden soil	Not indicated	Weight loss	64.3	180	(Jain and Tiwari, 2015)
PHB/CAB (50 %/50 %)					31.5		
PHB PHB/sugarcane bagasse (10 %) PHB/sugarcane bagasse (20 %) PHB/sugarcane bagasse (30 %)	Films	Compost Soil	Not indicated	ASTM G160-98 (weight loss)	87.8 86.7 88.4 84.5	77 119 112 84	(Sabapathy et al., 2023)
PHB PHBV (8 wt.% valerate)	Films	Soil	100 % relative humidity room temperature pH 6.6–6.7	-	100	14	(Kim et al., 2023)
PHB PHB/epoxidized natural rubber (70 %/30 %) PHB epoxidized natural rubber (70 %/30 %)	Films	Soil burial test	Not indicated	Weight loss	34.10 ± 4.37 3.93 ± 0 .83 5.20 ± 0 .39	28	(Chanasit et al., 2023)
PHB	Films	Garden soil	pH 7.45	Weight loss	73	21	(Chathalingath et al., 2023)
PHA PHA/Rice Husk (60/40 wt %) PHA-g-AA/Rice Husk (60/40 wt %)	Films	Alluvial-type soil	35 % soil moisture	Weight loss	35 > 90 84	60	(Wu, 2014)

PHBV (HV of 12; 43; 47; 52; 64; 72 mol %)	Powder	Soil/compost (90 % / 10 %)	25 °C, 65 % humidity, 80 % soil moisture holding capacity	CO_2, ASTM D5988-03	67; 54; 48; 62; 49; 49;	112	(Arcos-Hernandez et al., 2012)
PHA	Films	43 % certified organic topsoil, 43 % no-till farm soil, and 14 % sand	20 °C, 60 % moisture	CO_2, ASTM D5988-03	74.2	660	(Gómez and Michel, 2013)
PHBV/WF (50/50 wt %) PHBV/WF (80/20 wt %)	Films	Soil sub-tropical	80 % of soil water holding capacity	CO_2 ASTM D5988-03	36 35	330	(Chan et al., 2019)
PHBV PHBV / wheat straw	Films	Garden soil	23 °C, 21 % soil humidity	Weight loss	~20 ~23	180	(Avella et al., 2000)
PHB PHB + Acrylate	Films	Garden soil	Not described	Weight loss	100 10	180	(Shrivastav et al., 2011)
PHBV PDLLA/PHBV/PEG (30/70/20 wt %)	Films	Garden Soil	Room temperature	Weight loss	3 18	30 30	(Wang et al., 2008)
PHB PHBV (HV of 10 %)	Films	Hardwood, Pinewood, Sandy, Clay, Loamy soil	28 °C, pH 3.9; 3.5; 6.5; 7.1; 3.3, 14-22 % water	Weight loss	77; 74; 88; 78; 93 67; 64; 90; 53; 69	200	(Mergaert et al., 1992)
PHB	Films	Garden soil	pH 7.3 ± 0.2	Weight loss	83	77	(Sabapathy et al., 2019)
PHBV PHBV/WF (80/20 wt %) PHBV/Sisal fiber (80/20 wt %)	Films	Organic compound humidified using poultry feces and plant-origin organic materials	pH 6, 40 % maximum humidity, nitrogen of 1 %, minimum organic material of 40 %, C:N maximum of 18:1	Weight loss	9.77 ± 2.77 25.55 ± 4.05 25.02 ± 8.23	90 60 60	(Casarin et al., 2017)
PHBV-F0 PHBV-SF PHBV-PRF PHBV (HV of 3 %)	Particles around 1-2mm	Soil park (2.3 wt % of organic matter, 16.85 wt % of clay, 26.85 wt % of lime, and 56.3 wt % of sand)	28 °C, pH 6.8, 80 % of the soil water retention capacity	CO_2, ASTM D5988-96	100 100 100 91	75 79 87 123	(Lammi et al., 2019)
PHB–starch (75/25 wt %) PHB–starch (60/40 wt %) PHB–starch (40/60 wt %)	Films	Garden soil	25 ± 2 °C, pH 6.8, soil water content 45 %,	Weight loss	50 – 60	14-21	(Erkske et al., 2006)
PHB PHBV PHB3HHx PHB/4HB	Films	The soil of the temperate zone of Siberia, with high total exchangeable bases (40.0–45.2 mequiv/100 g), and with nitrate nitrogen N-NO_3 6, P_2O_5 60, and K_2O 220 mg/kg soil	28 °C, pH 7.1-7.8 and 50 % soil moisture	Weight loss	93 100 100 100	35 28	(Volova et al., 2017)
PHB				Weight	32 – 31.6	35	

PHB/peat PHB/clay PHB/WF	Powder-Granules	Agro-transformed soil with 280 mg/kg of phosphorus and 250 mg/kg of potassium	25 °C; 50 % soil moisture content	loss	43,6-53.6 36 - 26 33 -23		(Thomas et al., 2019)
PHB	Films	Forest soil	28 °C; 37 °C; 60 °C	Weight loss	10.5 ± 1.4; 7.1 ± 0.7; 4.9 ± 0.3	25	(Kim et al., 2000)
		Sandy soil	28 °C; 37 °C; 60 °C		5.8 ± 0.4; 10.0 ± 1.2; 4.5 ± 0.5		
		Farm soil	28 °C; 37 °C; 60 °C		41.3 ± 3.7; 68.8 ± 4.8; 14.8 ± 1.2		
PHA (MirelTM)	Films	Soil from the experimental field in Spata	30 °C, 40 % water content 40 °C, 40 % water content	Biological oxygen demand	26.3 49.5	10 12	(Rudnik and Briassoulis, 2011b)
PHBV (HV of 10 %)	Films	Soil from the Nagoya University Farm and 2 % (w/w) of Farmyard manure	30 °C, pH (H_2O) = 6.2, 40 % water content total C = 1.2 %, total N= 0.l l %, 40 °C, total C = 1.2 %, total N= 0. 1l %, pH (H_2O) = 6.2, 40 % water content	Weight loss	50 40	10 17	(Nishide et al., 1999)
PHB	Films	Soil	28 °C 37 °C 60 °C	Weight loss	57.3 86.7 25.9	56	(Rehman, 2015)
PHBV (HV of 11 %)	Films	1:1 mixture of black soil and leaf mold for gardening	25 - 30 °C	Weight loss	~28	180	(Teramoto et al., 2004)
PHBV/untreated abaca fiber					~48	90	
PHBV/Aa-abaca fiber					~48	180	
PHBV 6.2 mol % HV content	Films	Garden soil 270.4 g/kg of organic matter, 35.6 % humidity and pH ($CaCl_2$) 5.1	23 °C	Weight loss	100	30	(Gonçalves et al., 2009)
PHB	Films	Clay soil	20 °C; 30 °C; 40 °C	Weight loss	19.7 ± 0.8; 38.7 ± 2.6; 36.5 ± 1.5	200	(Manna and Paul, 2000)
		Laterite soil	20 °C; 30 °C; 40 °C		21.7 ± 1.0; 35.7 ± 1.5; 34.0 ± 1.2		
		Saline soil	20 °C; 30 °C; 40 °C		13.9 ± 0.7; 43.5 ± 1.7; 39.0 ± 1.4		
		Sandy soil	20 °C; 30 °C; 40 °C		17.6 ± 1.0; 33.5 ± 1.3; 26.5 ± 1.3		
		Tarine soil	20 °C; 30 °C; 40 °C		16.6 ± 0.8; 23.9 ± 0.9; 20.6 ± 0.9		
PHB/4HB (4HB of 5 mol %)	Films	Garden soil	Room temperature, 20 % water content	Weight loss	54.38	60	(Wen and Lu, 2012)
PHB/4HB (4HB of 7 mol %)					69.69		
PHB/4HB (4HB of 10 mol %)					79.91		
PHB/4HB (4HB of 15 mol %)					93.39		
PHB/4HB (4HB of 20 mol %)					82.03		
	Films	Farm soil	Not indicated		15.68	480	

PHBV (HV of 5 mol %)		Infertile soil		Weight loss	1.12		(Wang et al., 2005)
PLA/PHBV (70/30 wt %)	Films	Fresh soil from the surface layer of an agricultural field	23–25 °C, soil supplemented with 10 mL of 0.1 % $(NH_4)_2HPO_4$ solution	CO_2, ASTM D5988-12	32	200	(Muniyasamy et al., 2016)
PHBV					35		
PHBV (HV of 6.2 %) / PP-co-PE (80/20 w/w)	Films	Soil rich in humus	60 % of humidity	Weight loss	100	180	(Rani-Borges et al., 2016)
PHBV (HV of 6.2 %) / PP-co-PE/add (80/1 9/1 w/w/w) (with pro-oxidant additive)					100		
PHBV	Films	75 g of soil, 10 g of thin expanded perlite, 20 mL of deionized water	28 °C ± 1 °C	Weight loss	67 ± 18	34	(Montagna, 2016)
PHBV/CNT (99/1 wt %)					57 ± 6		
PHBV/CNT (98/2 wt %)					40 ± 15		
PHB	Films	Agro-transformed field soil, village Minino, Krasnoyarsk Territory	28 °C and soil humidity of 50 %	Weight loss	13	35	(Prudnikova et al., 2017)
PHBV					82	28	
PHB3HHx					90	28	
PHB/4HB					97	21	
PHB/WF (WF content 0 wt %)	Films	Alluvial-type soil, from farmland topsoil before planting	20 % soil moisture	Weight loss	4	84	(Wu, 2006)
PHB-g-AA/ WF (WF content 0 wt %)					5		
PHB/WF (WF content 30 wt %)					28		
PHB-g-AA/WF (WF content 30 wt %)					24		
PHB/WF (WF content 50 wt %)					49		
PHB-g-AA/WF (WF content 50 wt %)					42		
PHB	Films	The vegetable experimental field under organic farming (Spain)	25 °C, 50 % of soil water-holding capacity	Weight loss	100	120	(Barragán et al., 2016)
PHB	Films	Commercial soil	30 ± 0.1 °C, under moisture-controlled conditions	Weight loss	~72	30	(Rizzarelli et al., 2015)
PHBV (HV of 24 %)					~40		
PHB/Eastar Bio blend (75/25 %) + 30 % WF	Films	Soil (fertilizer) organic compound)	40 % maximum humidity, pH 6, maximum C:N 18:1, N (minimum) 1 %, minimum organic matter	Weight loss	29.32 ± 4.58	90	(Casarin et al., 2012)
PHB/Ecoflex blend (75/25 %) + 30 % WF					13.98 ± 1.83		
PHB					19.17 ± 3.43		
PHB/Eastar Bio blend (75/25 %)					2.83 ± 0.23		

PHB/Ecoflex blend (75/25 %)					1.86 ± 0.22		
PHBV (HV of 4 %)	Films	garden soil	28 ± 2 °C, 15 % soil moisture content 28 ± 2 °C, 20 % soil moisture content 28 ± 2 °C, 25 % soil moisture content 28 ± 2 °C, 30 % soil moisture content	Weight loss	12 23.6 95 95	60 45	(Kulkarni et al., 2011)
PLA/PHB_b 90D sun PLA/PHB_b for original carbon black-filled foil PLA/PHB_w for original transparent foil	Foils	Soil from agricultural regions mixed with perlite in equivalent amounts	20 °C, pH 7.5	CO_2 ASTM D5988-12 and ISO 17556-2012	55 42 57	365	(Jeszeova et al., 2018)
PHB/OMWR (100/0 w/w)	Films	Red soil - white soil		Weight loss	12.0 - 15.0	56	(Carofiglio et al., 2017)
PHB/OMWR (70/30 w/w)		Red soil - white soil	pH 8.2 - 8.3		21.9 - 22.6		
PHB/OMWR (60/40 w/w)		Red soil - white soil			24.6 - 26.2		
PHB	Films	Soil from Kolkata municipal solid-waste landfill (T1; T2; T3; T4; T5)	30 °C, pH 6.17, total phosphorous 0.79 %, total nitrogen 0.18 %, organic carbon 3.42 %	Weight loss	~12; ~19; ~12; ~15; ~10	28	(Dey and Tribedi, 2018)
PHB	Films	Paleudult soil	pH 6.17	Weight loss	100	180	(Schröpfer et al., 2015)
PHB3HHx	Films	Soil composted in the farm of Chubu University	34 °C, pH 5.3, relative humidity of 90 %	Weight loss	1.91 - 7.41	28	(Baidurah et al., 2019)
PHA	Films	Alluvial-type soil obtained from Taiwan Kaohsiung farmland topsoil	30 - 40 % soil moisture	Weight loss	25	60	(Wu et al., 2017)
PHA-g-MA					~30		
PHA-g-MA/TPLF (20 % w/w)					~65		
PHA-g-MA/PF (20 % w/w)					~70		
PHA-g-MA/TPLF (40 % w/w)					~85		
PHA-g-MA/PF (40 % w/w)					~90		
PHA	Films	Alluvial soil obtained from farmland topsoil before planting	35 % soil moisture	Weight loss	33	140	(Wu, 2013)
PHA-g-MA					35		
PHA-g-MA/TPF (20 % w/w)					75		
PHA-g-MA/t - TPF (20 % w/w)					66		
PHA-g-MA/TPF (40 % w/w)					88		
PHA-g-MA/t-TPF (40 % w/w)					84		

PHA	Powder	Natural soils, collected from the surface layer of one field and two forests or soil (15 g, plough layer, haplic chernozem; dry weight 88.4 %; soil texture, silty loam, volatile solids 5.55 %; soil organic matter 3.05 %)	25 °C	CO_2 ISO 17556 (2019)	85.8 - 96.4	150 - 170	(Šerá et al., 2020)
			37 °C		71.1 - 93.0	90-170	
PHB	Films	Artificial soil according to ASTM G 160-03	ASTM G 160-03	Weight loss	17.8 ± 0.64	86	(Mesquita et al., 2016)
PHB/PP-g-MA/clay (92 % / 5 % / 3 %)					22.5 ± 0.24		
PHB/PP-g-MA/clay (94.5 % / 2.5 % / 3 %)					25.9 ± 0.67		
PHBV	Films	Red clay latosol soil from a 0 to 15 cm depth profile	28 °C, 60 % of the moisture capacity	Weight loss	10	28	(Gonçalves et al., 2018)
PHBV-AgNP					8		
PHB	Films	Natural soil from agricultural land of the clay-loam type (clay 29 %, silt 28 %, and sand 43 %)	25 °C, C:N = 8, pH 7.9, water holding capacity 80 %, total nitrogen 2 %, organic carbon 0.13 %	CO_2, ISO 17556 2019	~88	120	(Briassoulis et al., 2020)
					~97	360	
	Powder	Natural soil mixture from three sources: sandy and forest	25 °C, C:N = 8, pH 7.9, water holding capacity 53 %, total nitrogen 7.9 %, organic carbon 0.51 %		~90		
		Standard soil: Prepared according to ISO 17556:2012	25 °C, C:N = 10, pH 8.4, water holding capacity 50 %, total nitrogen 3.7 %, organic carbon 0.22 %		~86		
		Natural soil mixture from three sources: sandy and forest	25 °C, C:N = 11, pH 7.4, water holding capacity 80 %, total nitrogen 6.8 %, organic carbon 0.31 %		~95	120	
		Natural soil: Sandy loam (Sand 62 %, Silt 27 %)	28 °C, C:N = 52, pH 7.6, water holding capacity 60 %, total nitrogen 4.2 %, organic carbon 0.03 %		~98		

Abbreviations: Wood flour (WF), 4-hydroxybutyrate (4HB), 3-hydroxyhexanoate (3HHx), olive pomace stone-rich fraction (SF), olive pomace pulp-rich fraction (PRF) olive pomace crude pomace (F0), anhydride-treated (AA-), polyhydroxybutyrate-co-hydroxyhexanoate) (PHB3HHx), maleic anhydride-grafted polyhydroxyalkanoate (PHA-g-MA), coupling agent-treated palm fiber (TPLF), palm fiber (PF), tea plant fiber (TPF) treated (crosslinked) tea plant fiber (t – TPF), carbon nanotubes (CNT), cellulose acetate butyrate (CAB), silver nanoparticles (AgNP), polypropylene-co-polyethylene (PP-co-PE), olive mill wastewater (OMW),

poly(ethylene glycol) (PEG), poly(d,l-lactide) (PDLLA), PLA/PHB outdoor weathering foil exposed 90 days to sunlight (PLA/PHB_b 90D), graphitized polypropylene with maleic anhydride (PP-g-MA), acrylic acid-grafted polyhydroxyalkanoate (PHB-g-AA), acrylic acid-grafted polyhydroxyalkanoate (PHA-g-AA)

PHB tested in different laboratories using the ASTM D5988 and some specifications of the ISO 17556 for plastics exhibit a similar degree of biodegradation in both natural and standard soils (Table 2.3). Other polymers that resist more to biodegradation, such as polybutylene terephthalate co-sebacate (PBSeT) and polybutylene sebacate (PBSe), present some differences, being slowly biodegraded in natural soils (Briassoulis et al., 2020). In another work, where the ASTM D5988 system was used, PHA biodegradation results were significantly different between two laboratories (Šerá et al., 2020). These authors also discovered that PHA degraded faster at 25 °C than at 37 °C in both laboratories. However, this may be due to higher biomass build-up and consequent carbon retention (Šerá et al., 2020). Kim et al. [58] demonstrated that the biodegradation of PHB was higher in different soils at 37 °C than at 28 °C, and the worst temperature for biodegradation was the highest tested, 60 °C (Kim et al., 2000). PHBV degraded at a faster rate at 30 °C than at 52 °C in soil under aerobic conditions (Nishide et al., 1999). Mergaert et al., (1993) discovered that the biodegradation rate in soils (laboratory testing) of PHB and PHBV (10 mol % HV) was enhanced at higher temperatures (40 °C), and similar results were obtained by other authors (Mergaert et al., 1992). The differences among these studies may be related to different microbial activities in soil, which are strongly influenced by temperature.

2.4.2 Microorganisms degrading PHAs

Microorganisms are capable of decomposing PHAs and can then use the resulting products of degradation as energy and carbon sources (Shrivastav et al., 2011). Bacteria identify these polymers as a source of organic compounds and energy. PHAs can be degraded by intracellular depolymerase enzymes when they are stored (Lee and Choi, 1999). Usually, PHB is degraded by a depolymerase that starts the hydrolysis originating free D(-)-3-hydroxybutyrate, which is then oxidized at acetoacetate by a NAD-specific dehydrogenase. NADH, pyruvate and 2-oxoglutarate inhibit this enzyme. The acetoacetate is finally converted to acetoacetyl-CoA by an acetoacetate/succinate CoA transferase. Acetoacetyl-CoA is at the same time a precursor of PHB synthesis and a product of degradation (Dawes, 1988).

Several microorganisms can produce and release extracellular enzymes that are effective in the degradation of PHAs (Shimao, 2001). Mergaert and Swings (1996) identified 695 microbial species capable of degrading PHB. PHAs are degraded by PHA hydrolases and PHA depolymerases (Choi et al., 2004). PHAs can be released into the environment due to the death and cell lysis of PHA-accumulating

cells or PHB plastic pollution. In the first step (biodeterioration) the microorganisms colonize the surface of the polymer, forming the biofilm (mixture of microorganisms, water and extracellular polymeric substances) (Ali et al., 2021). Because most of the polymers are too big to go through the microorganism's membrane, it is necessary the cleavage of polymeric molecules reducing gradually their molecular weight. (Ali et al., 2021). In the depolymerization step the adsorption of the binding domain of the PHAs depolymerase on the surface of the polymer occurs, followed by the hydrolysis of the chains by the catalytic domain of the enzyme (Hiraishi et al., 2010). The binding domain is non-specific; however, the active site of the catalytic domain is specific for the hydrolysis of the PHA film (Kasuya et al., 1996). Besides the domains, a linker region exists connecting the two domains (Hiraishi et al., 2010)**.** The polymer chain scissions may occur through endo-scissions (randomly throughout the chain) originating oligomers and then by exo-scissions (from the chain ends) originating only monomers (Kim et al., 2002; Scherer et al., 1999). Then the molecules are assimilated by the cells and the intracellular enzymes metabolize the polymer and use it as a source of nutrients and carbon. In aerobic conditions, PHAs are degraded to CO_2, biomass, and water as represented in Figure 2.5, and in anaerobic conditions, to water, biomass, CO_2 and methane. The process is called mineralization when an organic substance (such as polymers) is converted to an inorganic compound (Gu, 2003).

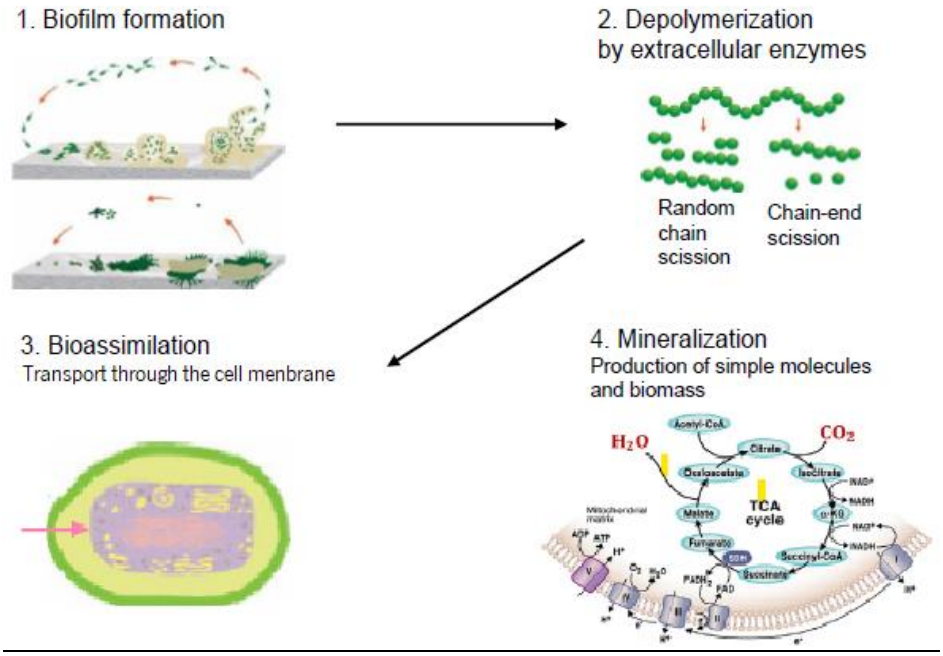

Figure 2.5 The four main stages involved in the biotic degradation process: (1) biofilm formation – establishment of microbial colonies on the polymer surface through the secretion of extracellular polymeric substances, (2) depolymerization – breakdown of polymer chains into small molecules such as oligomers, trimers, dimers, and monomers by the action of extracellular enzymes, (3) bioassimilation

— metabolization of low Mw compounds (dimers, monomers) by transportation through the cell membrane and (4) mineralization — carbon is biologically oxidized to CO_2 through a series of cycles, releasing energy and water and other compounds. Adapted from (Bher et al., 2022).

Microorganisms are the main responsible for the biodegradation of PHA-based plastics in several ecosystems. Biodegradation depends on the existence of PHA degrading enzymes (PHA depolymerases) produced by microorganisms that can hydrolyse water-insoluble PHA into water-soluble forms, so it can be used by these microorganisms (Sudesh et al., 2000). In soil, differences in the rate of PHA biodegradation can be due to several factors, including the composition of the microbial communities and to type and specificity of the depolymerases that they produce (Volova et al., 2017).

Almost 600 PHA depolymerases with different substrate specificities have been identified in various microorganisms (Knoll et al., 2009). Among intracellular and extracellular depolymerases and through the analysis of their sequences, they were classified into 8 superfamilies and 38 homologous families (Knoll et al., 2009). Several bacteria capable of PHA biodegradation are assigned to genera: *Stenotrophomonas, Alcaligenes, Comamonas, Rhodococcus, Rhodocyclus, Variovorax, Acinetobacter, Pseudomonas, Bacillus, Xanthomonas, Syntrophomonas, Ilyobacter,* and *Ralstonia* (Boyandin et al., 2012a; Jendrossek et al., 1996; Mergaert and Swings, 1996).

In several studies, fungi were able to biodegrade PHA polymers and were the dominant microorganisms colonizing the surface of the polymer (Sang et al., 2002; Šerá et al., 2020)**.** Fungi have higher biodegradation capability compared to bacteria because their PHA-depolymerases have higher mobility (Sultanpuram et al., 2008). Several groups of fungi, including *Ascomycetes*, *Basidiomycetes* and *Deuteromycete*s (e.g., *Penicillium simplicissimum*) zygomycetes, and micromycetes (e.g., *Penicillium, Paecilomyces, Acremonium, Verticillium,* and *Zygosporium),* among others, have been identified as capable of degrading PHA polymers (Boyandin et al., 2012a; Matavulj and Molitoris, 1992).

A rough surface allows better adhesion of the microorganisms and water, which accelerates the biodegradation of the polymers. A smooth surface decreases adhesion, which delays the biodegradation process (Mousavioun et al., 2012). The lag phase preceding the biodegradation of PHA films in soil may take days, weeks, or even months, depending on the composition and shape of the PHA and the environmental conditions. This lag period is the time needed for microbial adhesion to the material's surface and for the expression and release of extracellular depolymerases (Volova et al., 2017). Usually, a higher degree of crystallinity decreases microbial degradation, while the amorphous regions are easily

degraded (Nishida and Tokiwa, 1993b). In analogous ecosystems, in different regions, the biodegradability of PHBV is related to the number of PHBV degraders and is dependent on the growth conditions for PHBV degraders (Akmal et al., 2003; Muniyasamy et al., 2016; Song et al., 2003). Soil microcosms with higher functional diversity present better PHA biodegradation capacity than soil microcosms with lower functional diversity (Lim et al., 2005; Schröpfer et al., 2015).

Interestingly, some microorganisms can degrade several types of PHA in soil, but others can only degrade a specific PHA type. Volova et al. (2017) used the clear zone technique together with molecular-genetic methods (rRNA gene sequence) and found that PHB was degraded by bacteria of the genera *Mitsuaria*, *Chitinophaga*, and *Acidovorax*, but they were not detected on the surface of the copolymers PHB/4HB, PHB3HHx, and PHBV. *Roseateles depolymerans*, *Streptomyces gardneri*, and *Cupriavidus* sp. were specific degraders of PHB/4HB, *Roseomonas massiliae*, and *Delftia acidovorans* degraded PHBV, and *Pseudoxanthomonas* sp., *Pseudomonas fluorescens*, *Ensifer adhaerens*, and *Bacillus pumilus* degraded specifically PHB3HHx and *Streptomyces* were capable of degrading all PHA polymers (Volova et al., 2017). Some microorganisms can produce several types of depolymerases, and thus have a broader range of PHA biodegradation potential, while others only produce one kind of depolymerase capable of PHA biodegradation (Schirmer et al., 1995).

2.4.3 PHA blends and/or composites

The cost of bio-based plastics is still higher than that of the plastics produced from petroleum raw materials. Furthermore, some have lower barrier properties, or flexibility (which makes them less suitable for food packaging) (Nguyenhuynh et al., 2021; Teixeira et al., 2020). The use of PHAs (Figure 2.6) with other polymers (blends) or with naturally decomposable materials such as natural fibers (composites), is a strategy to reduce production costs or change the properties according to the goals established (Shrivastav et al., 2011).

Composite films produced with PHBV and reinforced with *Ceiba pentandra* bark fibers. (5 % - 20 %) demonstrated enhanced mechanical and thermal stability when compared with the neat PHBV. The composite films were tested as packaging material for fruits and were able to maintain freshness after 7 days when compared with unpacked fruits (Varghese et al., 2020). A composite of PHB with 10 % and 20 % coconut fibers presented increased elastic modulus and stiffness compared only with the PHB matrix (da Silva Moura et al., 2019). Composites of PHB/cotton fibers with an epoxy coupling agent presented the highest Young's modulus, tensile strength, and strain at maximum stress than PLA and cotton fibers composites (Battegazzore et al., 2018).

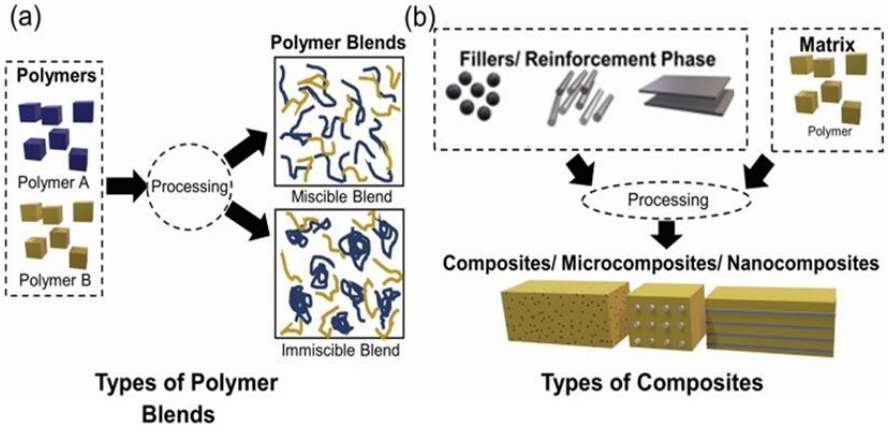

Figure 2.6 Illustration of polymer composites and blends. (a) The mix of two or more polymers originates miscible/immiscible blends. (b) The reinforcement phase is combined with a polymer matrix to produce composites. Adapted from Toh et al., (2021).

In other works, the coupling of PHAs with other polymers was explored. Bianco et al., (2013) studied PHBV blends with polyethylene oxide (PEO) fibrous mats. The blends presented increased viscosity and thermal and with the increase of PEO content but decreased fiber size. Blends of PCL with PHBV were prepared and then used to produce 3D scaffolds. The increase in the roughness and hydrophilic nature of the blends improved the cellular functions, demonstrating the potential of the scaffolds to be used for cartilage tissue engineering purposes (Kosorn et al., 2017). PHB/PVA blends with different proportions presented different behaviours and with 20 % to 30 % PHB content. The mechanical properties and water diffusion coefficients switched significantly. The authors indicated that the films have the potential to be applied in the packaging, agricultural, and biomedical industries (Ol'khov et al., 2015). However, this process also changes the biodegradation properties and requires a new assessment of the material biodegradability. Some blends can be used to change the properties of PHA, but these blends can also change the biodegradation behaviour of PHA, increasing or decreasing the biodegradation rate, depending on the material blended to the PHA [62,65,70,86,104-113]. These different effects can be the result of 1) an overall crystallinity change since crystalline zones are less accessible for the microorganisms, 2) microstructural defects that facilitate the adhesion of bacteria, or even 3) increased hydrophilicity of the blends that facilitate water adsorption that is fundamental for the microorganism biodegradation (Lammi et al., 2019; Wei et al., 2015b). The biodegradation behaviours of PHB/lignin blends (films) were analysed in a soil field study. The results indicated that PHB films disintegrated with 45 % of weight loss within 12 months, however, the PHB/lignin blends had only a weight loss of 12 % when 10 % of lignin

was present. These results suggest that the presence of lignin can reduce the PHB biodegradation, probably by hampering the colonization by microorganisms (most likely due to its hydrophobicity), which improved the resistance of the blends to microbial activity (Mousavioun et al., 2012).

Jeszeova et al., (2018) tested PLA/PHB foils with the ASTM D5988 biodegradation test (CO_2 production), and the PLA/PHB white foils showed the best biodegradation (57 %), followed by the PLA/PHB black foils previous exposed to outdoor weathering for 90 days (55 %) and finally by the PLA/PHB black foils (42 %). The microorganisms present in the soil, and potentially involved in the biodegradation of the films, were identified by using culture-dependent methods (i.e., microbial strains were cultivated in three different growth media containing PLA/PHB blend, PHB or PLA) and culture-independent methods (i.e., 16S rRNA gene-based diversity analysis by denaturing gradient gel electrophoresis (DGGE) and cloning). This strategy allowed the identification and isolation of several PLA/PHB blend degrading microorganisms assigned to several genera, including *Bacillus*, *Streptomyces*, *Rhodococcus*, *Saccharothrix*, *Fusarium*, *Trichoderma*, and *Penicillium*.

The biodegradation of PHBV/wood flour (WF) was more significant in the laboratory (PHA50WF -35 %, PHA20WF – 36 %) using ASTM D5988 than in the field study. Wu, (2014) explained this result as a consequence of the higher moisture content of the soil laboratory test, which has been shown to accelerate the biodegradation of both wood and PHA. But contradictory results were obtained, demonstrating higher biodegradation of PHB in garden soil than in laboratory settings (using the same soil) (Wang et al., 2008). As previously indicated, the temperature can significantly influence the biodegradation process and PLA/PHA mulches degraded more extensively in the soil during the summer than in wintertime because the warmer temperatures promoted microbial activities (Jendrossek et al., 1996).

2.4.4 PBAT biodegradation in soil

PBAT is only considered compostable under typical compost conditions (Witt et al., 2001). It is considered very resistant to biodegradation in natural environments such as soil (Han et al., 2021). However, some studies, report PBAT biodegradation in soil. For example, pristine PBAT films could be partially biodegraded in soil but at a relatively slow rate (e.g., 21 % in 180 days, 18 % in 180 days, and 6 % after 100 days) when compared with PHAs biodegradation (Palsikowski et al., 2018; Šerá et al., 2016; Souza et al., 2019). Even though the degradation of PBAT is considered slow, especially at mesophilic temperature, it must be pointed out that Wang et al. (2015) noticed that antimicrobial PBAT composite films buried in soil remained biodegradable. The biodegradation in real soil, evaluated by mechanical

properties instead of laboratory soil tests, is significantly faster, being 1 month in the field, equivalent to 6 months in the laboratory, in terms of mechanical changes (Nikolić et al., 2017).

The microbial community is one fundamental component of the polymer's biodegradation, and soils have a great variety of microorganisms capable of producing extracellular enzymes. Thus it is possible that suitable enzymes can be produced by several species for the same role in the biodegradation process (Šerá et al., 2020). Nonetheless, Kijchavengkul et al. (2010a) found that the PBAT biodegradation in compost was different, even respecting the recommended parameters of the standards. According to the authors, this may be due to the lack of uniformity of microbial composition among and within the composts. This indicates different enzymatic preferences of microorganisms for different isomers, ignoring the specific microorganism's community's presence, which may be more or less effective than others at biodegrading the polymer studied.

Several strains of *Bacillus* and *Actinomycetes* have been identified in soil or compost as capable of degrading PBAT (Kanwal et al., 2022; Witt et al., 2001). The relative abundance of *Bacillus* tends to increase during the biodegradation assays (Kanwal et al., 2022; Witt et al., 2001). The existing scientific knowledge indicates the strong possibility that PBAT degradation in soil is the result of the synergistic activity of different organisms. This was verified, for example, by Šerá et al. (2020), in which the consortium that biodegraded was formed by *Thermobispora bispora* (a thermophilic actinomycetes) and *Bacilli* species, which could not carry out the biodegradation alone. The isolation may deprive the microorganisms of their necessary interactions and synergetic activity to accomplish the biodegradation. The diversity and composition of the soil community may thus influence biodegradation because other microorganisms, might not have enzymes capable of degrading directly PBAT, but may contain enzymes involved in the downstream pathway of degradation, and able to catabolize intermediates of PBAT degradation, thus acting synergistically. PBAT films were found to be degraded at different rates in manure, food, and yard compost (Kijchavengkul et al., 2010b). In lou, fluvo-aquic, black and red soil, differences in the biodegradation of films were obtained, due to microbial community differences (Han et al., 2021). Šerá et al. (2020) found no mesophilic PBAT degraders in 41 temperate zone soils and found a low number of thermophilic degraders in only nine soils, although some more were found, after an enrichment process. This indicates that the complete biodegradation of blends or composites with PBAT in their composition may be slow due to the shortage of microorganisms able to degrade it. In short, it is crucial to study these materials, because some formulations can be degraded at a faster rate than pristine PBAT films (Šerá et al., 2016).

2.4.5 Microorganisms degrading PBAT

The enzymatic hydrolysis of PBAT can produce bis(4-hydroxybutyl) terephthalate and 1,4-butanediol that results from the hydrolysis of the aliphatic ester bonds (butylene adipate), as well as mono(4-hydroxybutyl) terephthalate and terephthalic acid resulting from the hydrolysis of the aromatic ester bonds (butylene terephthalate) (Müller et al., 2017). Several routes have been identified for the PBAT and PBAT monomers biodegradation. Terephthalic acid can be aerobically degraded using different degradation pathways. Normally it is converted into protocatechuate, via the metabolic funnel of aromatics that needs only little dedicated enzymes as represented in Figure 2.7 (Wang et al., 1995). Then the protocatechuate is converted into pyruvate and further to acetyl coenzyme A (Daisuke et al., 2009). 1,4-butanediol can also be oxidized to 4-hydroxybutyrate and then later to succinyl-CoA by oxidation, although it can be oxidated to succinate or beta-oxidated to glycolyl-CoA and acetyl-CoA (Li et al., 2020). Adipic acid can also be converted to adipyl-CoA by an adipate-CoA ligase and then further converted to acetyl-CoA (Strittmatter et al., 2022). The products resulting from the monomers can be introduced into the tricarboxylic acid cycle to create energy, and release CO_2 and water (Bher et al., 2022).

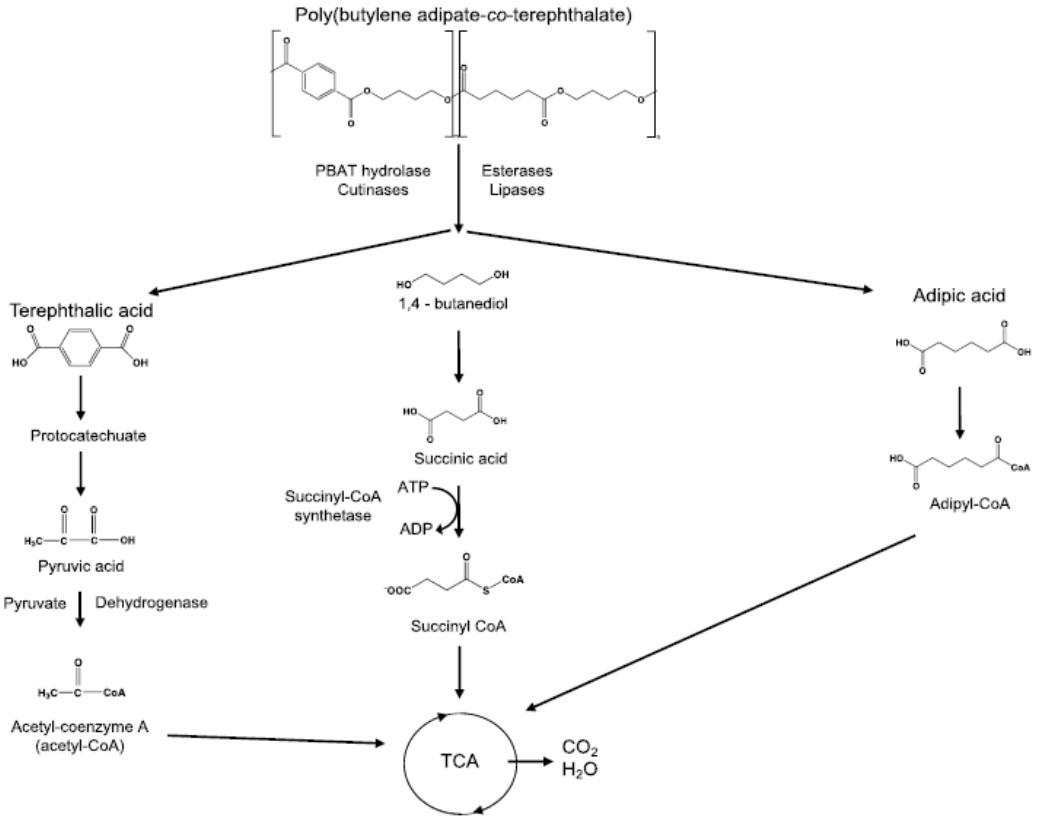

Figure 2.7 Common aerobic biodegradation pathway for PBAT, starting from the hydrolysis by esterases and lipases and ending on the Tricarboxylic acid cycle (TCA) cycle (Bher et al., 2022).

Normally, the biodegradation of the aliphatic ester bonds is faster, since they are soft and amorphous, contrary to the more rigid and crystalline aromatic ester bonds (Fu et al., 2020). Even so, not many microorganisms have been identified as able to degrade PBAT, but most of the strains belong to 3 phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Table 2.4 shows the organism identified as capable of degrading PBAT. At thermophilic temperatures, *Thermomonospora fusca* and *Thermobifida alba* have been identified as initial degraders (Hu et al., 2010; Kleeberg et al., 1998; Thumarat et al., 2012, 2015). At mesophilic temperatures, *Bacillus subtilis, Bacillus pumilus*, *Leptothrix sp. TB-71, Peribacillus frigoritolerans* S2313*, Stenotrophomonas sp.* YCJ1 (isolated from farmland soil) *Rhodococcus* strain NKCM 2511 and *Rhodococcus fascians NBRC 100625* were the aerobic bacteria that presented the capacity to degrade PBAT (Jia et al., 2021; Muroi et al., 2017; Nakajima-Kambe et al., 2009a; Soulenthone et al., 2020; Trinh Tan et al., 2008; Wufuer et al., 2022). A bacterium, *Roseateles depolymerans* TB-87, isolated from freshwater was reported as able to degrade aliphatic and aliphatic-aromatic copolyesters, including PBAT (Shah et al., 2013a, 2014).

Table 2.4 Organisms and enzymes identified as capable of degrading PBAT

Microorganism	Enzyme	NCBI ID	GenBank/ UniProt/MGnify	EC number	PDB entry	References
Pseudomonas pseudoalcaligenes	Polyesterase	WP_003460012	W6R2Y2	EC 3.1.1.74		(Wallace et al., 2017)
Rhodococcus fascians NKCM251	Hydrolase	BCL64964	A0A7I8E2Z4			(Soulenthone et al., 2021)
Thermobifida cellulosilytica	Cutinase	ADV92526	ADV92526.1	EC 3.1.1.74	5LUI	(Perz et al., 2016a)
	Cutinase	ADV92527			5LUI	
Thermobifida alba	Cutinase	BAI99230	BAI99230	EC 3.1.1.1.74		(Thumarat et al., 2015)
Thermobifida alba	Cutinase	BAK48590	F7IX06	EC 3.1.1.1.74		(Thumarat et al., 2012)
Pelosinus fermentans DSM 17108	Hydrolase	AIX10936	EIW29778.1			(Biundo et al., 2016)
Clostridium botulinum	Esterase	AKZ20828	AKZ20828.1			(Perz et al., 2016b)
	Esterase	AKZ20829	AKZ20829.1			
Bacillus pumilus	Hydrolase	BAV72205	A0A1E1FNX8			(Muroi et al., 2017)
Saccharomonospora viridis	Polyesterase	WP_015787089 /BAO42836				(Kawai et al., 2014)
uncultured bacterium	Carboxylesterase	AOR05748				(Müller et al., 2017)
uncultured bacterium	Carboxylesterase	AOR05749				

uncultured bacterium	Carboxylesterase	AOR05750				
uncultured bacterium	Carboxylesterase	AOR05751				
uncultured bacterium	Carboxylesterase	AOR05752				
uncultured bacterium	Carboxylesterase	AOR05753				
Humicola insolens	Cutinase	QAY29138	A0A075B5G4	EC 3.1.1.74	4OYL	(Perz et al., 2016a)
Fusarium solani	Cutinase	AAA33334		Novozym® 51032		(Zumstein et al., 2017)
Rhizopus oryzae	Lipase	Organism (AB721967)/1TIC		sigma 80612/62305		(Zumstein et al., 2017)
Leptothrix sp. strain TB-71	Esterase	Organism (AB458235)				(Nakajima-Kambe et al., 2009; Shah et al., 2014)
Hungatella hathewayi DSM 13479	Esterase	ALS54749				(Perz et al., 2016c)
Thermobifida fusca DSM 43793	Hydrolase	CAH17554				(Kleeberg et al., 2005; Müller et al., 2005)
	Hydrolase	CAH17553				
Paraphoma sp. B47-9	Cutinase	BAN51853				(Suzuki et al., 2014)
Saitozyma flava (Cryptococcus flavus)	Cutinase	BAT32793				(Watanabe et al., 2015)
Stenotrophomonas sp. YCJ1 (lipase)						(Jia et al., 2021)
Roseateles depolymerans TB-87						(Shah et al., 2013a, 2014)
Marinobacter sp.,			OK558824		7VPA	(Meyer-Cifuentes et al., 2020)
			OK558825		7VMD	
Isaria fumosorosea NKCM 1712						(Kasuya et al., 2009)
Cryptococcus sp. MTCC 5455						(Aarthy et al., 2018)
Peribacillus frigoritolerans S2313						(Wufuer et al., 2022)
Bacillus subtilis						(Trinh Tan et al., 2008)

Even in anaerobic environments, PBAT is biodegraded, although at a lower rate than in aerobic environments. The bacterium *Hungatella hathewayi DSM 13479* (formerly known as *Clostridium hathewayi*) was found as capable of degrading PBAT anaerobically (Perz et al., 2016c). Two PBAT hydrolases were also identified from the anaerobic mesophilic bacteria *Clostridium botulinum* ATCC 3502 and *Pelosinus fermentans* DSM 17108 (Biundo et al., 2016; Perz et al., 2016b).

Some strains of yeast and fungi have also been indicated as PBAT-degrading organisms, namely *Cryptococcus sp. MTCC 5455* (phylum Basidiomycota), *Isaria fumosorosea NKCM 1712* (phylum Ascomycota), Paraphoma-related fungus cutinase-like enzyme, and *Cryptococcus flavus* cutinase-like enzyme (Aarthy et al., 2018; Kasuya et al., 2009; Suzuki et al., 2014; Watanabe et al., 2015). *Rhizopus oryzae* lipase and *Fusarium solani* cutinase are enzymes capable of degrading PBAT films with different terephthalate-to-adipate molar ratios (Zumstein et al., 2017). A fungal cutinase from *Humicola insolens* (HiC) and a bacterial cutinase from *Thermobifida cellulosilytica* (Thc_Cut1) were also able to hydrolyse the PBAT-tested esters (Perz et al., 2016a). About 6 enzymes of uncultured microorganisms in microbial communities associated with the *Sphagnum magellanicum* moss, were able to degrade PBAT (Müller et al., 2017).

In aquatic environments, that are not as well studied, a PBAT-hydrolysing enzyme has been described from the *Pseudomonas pseudoalcaligenes*, a typical aquatic microorganism (Wallace et al., 2017). In a marine microbial enrichment culture, two enzymes were also identified in species from the genus *Marinobacter sp.* (Meyer-Cifuentes et al., 2020). As indicated before, several types of enzymes are capable of degrading PBAT, namely, cutinizes, lipases and PBAT hydrolases (Aarthy et al., 2018; Jia et al., 2021; Kasuya et al., 2009; Soulenthone et al., 2021; Zumstein et al., 2018). Nonetheless, the extracellular enzymes identified as capable of degrading PBAT are quite scarce in comparison to those for common aliphatic polyesters. It is important to mention, that not all organisms or enzymes are capable of completely degrading all the components of PBAT, being the aliphatic unit much more susceptible to degradation than the aromatic unit. Some of these studies tested directly the efficiency of the enzymes, and very few study the PBAT degradation in real or simulated environments, such as soil, compost or water.

2.4.6 PBAT blends or Composites

The same type of strategy explained in section 2.4.3 can be applied with PBAT. Dammak et al. (2020) created a blend of PBAT with plasticized thermoplastic starch (TPS) and included suitable compatibilizers (e.g., maleic anhydride) for packaging applications. PBAT/TPS composites have lower prices but frequently present poorer mechanical properties (Liu et al., 2020b). PBAT/TPS blends with a compatibilizer (Joncryl-ADR-4368), containing synthesized styrene-maleic-anhydride-glycidyl methacrylate (SMG) reactive compatibilizers presented enhanced mechanical performances appropriated for several applications, such as packaging and agriculture mulching films (Wei et al., 2015a).

Another frequent polymer blended with PBAT is PLA, nevertheless, PLA/PBAT blends present multiphase behaviour because of the non-compatible attributes of each element, which produces weak mechanical properties (Sarath Kumara et al., 2008). Jiang et al., (2006a) reported alterations from brittle fracture to ductile fracture in tensile testing with increased PBAT fraction and low interfacial adhesion. PBAT/PLA films developed for agricultural purposes (mulch films), with 10-20 % in weight of calcium carbonate presented enhanced compatibility with good maximum strain, tensile strength and Young's modulus (Rocha et al., 2018). The addiction of Joncryl and 1,6-hexanediol diglycidyl ether to the blends has been reported to improve up to almost 500 % of the strain at break (Dong et al., 2013). Other well-known PBAT blends are created with lignin. Xiong et al. (2020) produced PBAT/lignin blends via melt extrusion with up to 60 % lignin content. Although the authors reported a cost reduction of about 36 %, the tensile (23.70 to 14.41 MPa) and elongation (816.49 % to 378.94 %) properties were also reduced. The addition of methylated lignin (60 %) to PBAT increased these properties in relation to the PBAT/lignin films, but they were still inferior to the pristine PBAT films (Xiong et al., 2020).

The development of new composites and blends also needs validation of the biodegradation behaviour, since if the properties of the initial materials can be changed, the biodegradation potential can also be affected. The biodegradation in soil of PBAT blends or composites is not as well studied as PHAs. PBAT/lignin blended films presented a decrease in mechanical properties after soil biodegradation when compared with neat PBAT films (Liu et al., 2021). Films of PCL blended with PBAT presented a superior biodegradation (evaluated by mass loss) after 119 days in soil (37 %) than PBAT films (2.3 %), however, the biodegradation was inferior to the PCL films (57 %) (Sousa et al., 2022). This test was conducted between 30–35 °C. The PCL incorporation did not affect negatively all the properties including the tensile properties, indicating the authors that the blends higher permeability can make the films useful in several applications. PBAT films with calcium carbonate ($CaCO_3$) nanoparticles as nanofiller, were incubated at 30 °C in soil. The $CaCO_3$ nanoparticles decreased surface wettability and hindered the disintegration. The SEM analysis after the soil test revealed selective zones of disintegration (Rapisarda et al., 2022). PLA/PBAT blends are a little more studied and sometimes fillers or compatibilizers are used to enhance the properties. Graphene-modified composite of PLA/PBAT presented different behaviours depending on the graphene content. The FTIR analysis indicated the priority degradation of PLA. With 0.2 % of graphene (in weight) the degradation was inhibited, but with 1.0 % the degradation increased (Liu et al., 2022b). The incorporation of montmorillonite clay in PLA/PBAT films resulted in a slower biodegradation in soil (28 °C), probably montmorillonite enhanced the hydrolysis by enabling water penetration but delayed the diffusion of oligomers to be used by microorganisms (Freitas et al., 2017). PLA/PBAT blends

compatibilized with a chain extender (Joncryl ADR-4368) exhibited an intermediate behaviour compared to neat polymers, the authors indicated an increased crystallinity in the blend, as one factor that influenced the biodegradation of the blends (Palsikowski et al., 2018).

2.5 Factors affecting plastic biodegradation

2.5.1 Environmental factors

When plastics are introduced in soil several factors can affect the biodegradation process (Figure 2.8). Soil is a diverse environment for microorganisms and biodegradation typically occurs in the mesophilic range of temperature (Nannipieri et al., 2017). On the surface, they are exposed to several abiotic factors such as sunlight, but in the soil underground matrix other factors related to the effective action of the microbial community, such as pH, are more important (Bastioli, 2005). The soil is classified into several groups. The soil granularity and porosity depend on the clay, sand (fine and coarse), and silt relative proportions (Ramos et al., 2017). The different soil particles create pores, where the water and nutrients are, and in this case, the soil structure and texture are other factors that explain differences in the communities due to spatial separation (Najmadeen et al., 2010). Soil water is one factor influencing the microorganism distribution since wet soils favour bacterial populations while in dry soils the fungi populations thrive (Bastioli, 2005)**.** The advantage of fungi is that they develop a network of thin filaments (hyphae) and can find water and nutrients in different places allowing growth and survival (Frey et al., 2000). The soil oxygen (O_2), also determines if the microbial population is aerobic or anaerobic, which limits the type of biodegradation possible. Soil pH can determine microbial activity. Alkaline conditions favours bacterial growth and in opposition, acid pH favours fungal growth (Johannes et al., 2009). In acidic pH, trace metals such as zinc, may have increased mobility, forming an aggressive environment for soil bacteria (Kicińska et al., 2022). The soil organic matter may also influence the distribution of microorganisms since they have a preferential carbon source (Kramer and Gleixner, 2008). The soil temperature also helps regulate chemical, physical, and biological processes. Changes in soil respiration occur due to variations in temperatures, being normally higher with superior temperatures (Qu et al., 2023). The microbial activity is normally low with lower temperatures (Mergaert et al., 1993). Finally, radiation is capable of reducing the growth of microbial populations, depending on the intensity (Silva et al., 2022). Some microorganisms even become more resistant such as *Deinococcus frigens* sp. nov., *Deinococcus saxicola* sp. nov., and *Deinococcus marmoris* sp. nov**.** (Hirsch et al., 2004; Rainey et al., 2005)

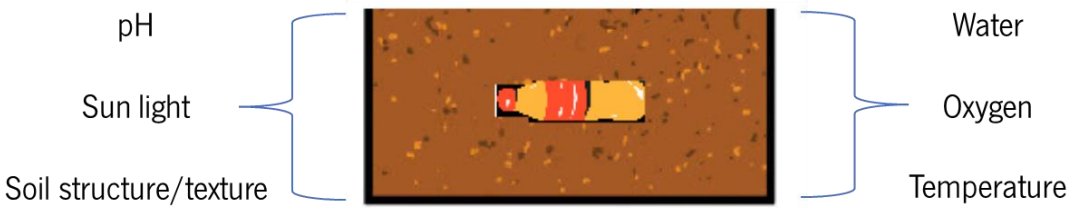

Figure 2.8 Environmental factors that influence polymer biodegradation. Adapted from Ali et al. (2021).

The environmental factors affect not only the microorganisms but also the polymer. UV light can cause photodegradation, originating crosslinking within the films which promotes brittleness (Kijchavengkul et al., 2008a). PHB submitted to photodegradation using artificial UV radiation presented an increased quantity of carbonyl groups, chain scission and crosslinking reactions, and a rougher surface, cracks and fissures were also found (Sadi et al., 2010). These changes can limit the biodegradation since the biodegradation of this PHB exposed to photodegradation was delayed due to an increase in the crystallinity (Sadi et al., 2010). The photodegradation has been also indicated for PBAT films resulting in a crosslinking effect, which slows down the biodegradation rate because it decreases the chain mobility of the polymer (Kijchavengkul et al., 2008a, 2008b, 2011). It is also known that PBAT suffers from mechanical degradation, namely erosion due to its main application in agricultural mulch films (Qi et al., 2021). Moisture can also cause the degradation of hydrolysable chemical bonds due to their susceptibility to chain scission reactions that can occur by depolymerization or random chain scission (Muthuraj et al., 2015). The hydrolytic degradation of PBAT is mainly due to hydrolysis of the ester linkages, which occurs essentially randomly along the backbone of the polymer chain. Water can also react with the carbonyl functions close to the benzene ring. Salguero et al., (2012) subjected PHB to an invitro, saline solution of phosphate (pH 7, 37 °C) for 40 days and found a decrease of about 10 % in the contact angle due to the hydrolysis producing hydrophilic chains end, causing a higher absorption of water. The temperature can cause chain mobility, and polymer rearrangements specialty at superior temperatures than the glass transition temperature (T_g) of the polymer which increases the rate of polymer biodegradation (Palsikowski et al., 2018). The molecular weights of PHB and PHBV incubated at 40 °C in soils decrease at similar rates to the same samples incubated at 40 °C in sterile buffer (Mergaert et al., 1993). Nevertheless the erosion rate in soil increased at higher temperatures, being in most of the tests, higher for the PHBV (Mergaert et al., 1993). Random β-C-H hydrogen transfer reactions in the PBAT are believed to occur also with thermal biodegradation (Al-Itry et al., 2012).

2.5.2 Polymers properties

Several properties of the films can also have a direct impact on the biodegradation process (Figure 2.9). Properties such as density affect liquid diffusion or hydrophobic/hydrophilic surfaces, which in the hydrophobic case delays water absorption (Gazvoda et al., 2021). The aliphatic chains in PBAT exhibit high biodegradation rates when compared with the PBAT aromatic rings (Marten et al., 2005). The flexibility and mobility of the PBAT aliphatic chains facilitate the binding of the enzymes (Marten et al., 2005). The chemical structure of the polymers (spatial organization) helps shape the geometry originating different regions (amorphous or crystalline) in PBAT films (Muroi et al., 2017). The PBAT crystallinity plays a major role, since normally crystalline regions are more rigid and stiff, and consequently are less prone to degradation, while the amorphous regions are more susceptible to hydrolysis (Kijchavengkul et al., 2010b). The molecular weight influences biodegradation, since the higher the molecular weight, the harder it is for microorganisms to bio-assimilate PBAT chain sections, which decreases the biodegradation rate (Kijchavengkul et al., 2010b). The size and shape of the polymers influence the surface area of the material, which is important since the biodegradation starts on the surface (Šerá et al., 2016). For example, PBAT with 25 % starch-containing biodegradable plasticizers, presented an increased biodegradation according to the authors due to an increase in the active surface area after the biodegradation of the filler (Šerá et al., 2016). PBAT and scl-PHA were among the most degradable polymers in uncontrolled composting conditions and all of them presented very rough surfaces as compared to the other polymers (Mercier et al., 2017). The roughness has an impact on biodegradation since irregularities provide places for microbial attachment, and therefore favour microorganisms-polymer contacts that may increase polymer degradation (Mercier et al., 2017).

Figure 2.9 Polymer characteristics that influence polymer biodegradation. Adapted from Ali et al., (2021).

Several studies have demonstrated that the properties of PHA materials can affect the biodegradation rate. Specifically, copolymers (polymers derived from more than one species of monomer) are degraded at a faster rate than homopolymers, although the differences varied widely between soils with several pH

values (neutral or acid) and temperatures. Copolymers (e.g., PHBV) usually have a higher degradation rate compared to homopolymers (e.g., PHB) of the PHA family (Mergaert et al., 1993). This higher biodegradation capability is attributed to the surface morphology of copolymers, which combines a low crystallinity and a porous surface, allowing faster degradation (Sridewi et al., 2006). The biodegradation of PHAs with different chemical compositions was tested in soil for 35 days. These polymers could be ordered as follows according to the biodegradation rate (from higher biodegradation rate to lower biodegradation rate): PHB/4HB > PHB3HHx > PHBV > PHB (Volova et al., 2017). PHB films, the most crystalline ones, remained nearly unchanged, suggesting that all regions (crystalline and amorphous) were degraded at similar rates (Volova et al., 2017). But for the 3 copolymers, the crystallinity increased, demonstrating that the amorphous regions were degraded at higher rates. Other works, including studies performed in natural environments (where the biodegradation was evaluated by weight loss), present the same pattern, indicating that PHB is more resistant to biodegradation due to its high crystallinity in comparison with the copolymers (Boyandin et al., 2012a; Prudnikova et al., 2017; Salim et al., 2012). The chain of the polymer also influences biodegradation, since crosslinking normally reduces the biodegradation of PHAs polymers (Zhao et al., 2006). The size of the chain may influence the enzymatic biodegradation, with longer chains in PHA polymer originating lower biodegradation (Li et al., 2007). Thicker PHA films were also harder to biodegrade than the less thick, this is also a characteristic that influences biodegradation (Ong and Sudesh, 2016).

In conclusion, the rate of biodegradation in soil is influenced by several factors, including the properties of the polymer such as crystallinity, surface area, composition, and shape, the environmental factors such as temperature, moisture level, pH, and nutrient supply, the microbial communities and the activity and specificity of microbial depolymerases. These factors interact, creating different soil environments and different biodegradation potentials from place to place, from season to season.

2.6 Types of tests to evaluate plastic biodegradation

Several methods exist to test the biodegradation of plastics. Each method has its advantages and disadvantages and can be divided into 3 groups, namely field tests, simulation tests, and laboratory tests as represented in Figure 2.10. Field tests are performed directly in the environment (e.g., soil, water stream). However, it is impossible to control the environmental factors (e.g., temperature, pH) at which the material is exposed and it is challenging to establish methods to evaluate biodegradation (Plackett and Katiyar, 2011). Typically, direct analysis, namely visual alterations in the polymer is used or weight

loss determination. In this last case, fragmentation of the material or adsorbed soil particles leads to error measurement (Müller, 2005). Simulation tests are conducted in laboratory-simulated conditions, (e.g., in bioreactors) that represent well real environmental conditions. The main difference is that several parameters including temperature, pH and humidity, can be controlled and/or adjusted, and the number and quality of analytical tools that can be used to assess biodegradation are better and include the evolution of CO_2 or O_2 consumption (Müller, 2005). Biodegradation tests in soils have been developed for evaluating the degradability of plastics (Šerá et al., 2020).

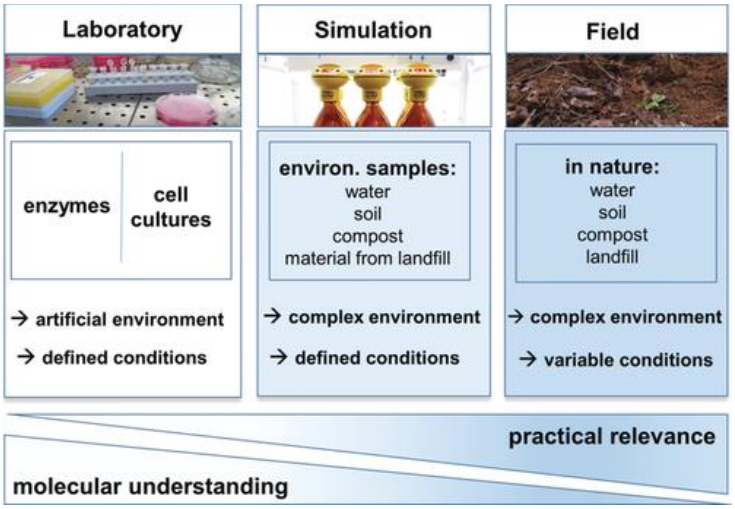

Figure 2.10 Scheme of the different biodegradable tests to analyse plastics Adapted from (Haider et al., 2019).

Finally, laboratory tests are highly reproducible, and generally defined media are used (e.g., synthetic media) and inoculated with a mixed microbial culture obtained from the environment or a specific microbial strain or enzyme (Müller, 2005). These tests can be optimized in relation to the activity of the microorganisms used, which frequently results in a better degradation rate than in natural conditions. They can be very useful to study the mechanisms of polymer biodegradation. However, in controlled conditions, only limited conclusions about the degradation rate of plastics in a real environment can be raised, so they are less relevant in this sense (Müller, 2005).

2.7 Techniques to evaluate and monitor biodegradation

Several types of methodologies, qualitative or/and quantitative can be used to determine biodegradation. Besides quantitative methodologies, such as CO_2 evolution and molecular weight reduction, qualitative methods, such as SEM and spectroscopy can be used to sustain and better understand the

biodegradation process (Figure 2.11). Some of the most common methodologies are weight or mass loss and assessment of mechanical properties, however, they indicate physical degradation but not the action of microorganisms. The clear zone technique and spectroscopy combined with chromatography among others can be used to detect enzymatic activity. The more precise techniques, and most recommended by standard methods, are the respirometric methods that track CO_2 evolution and mineralization or biochemical oxygen demand (BOD). The radiolabelling can also be used as an accurate method to track carbon from polymers.

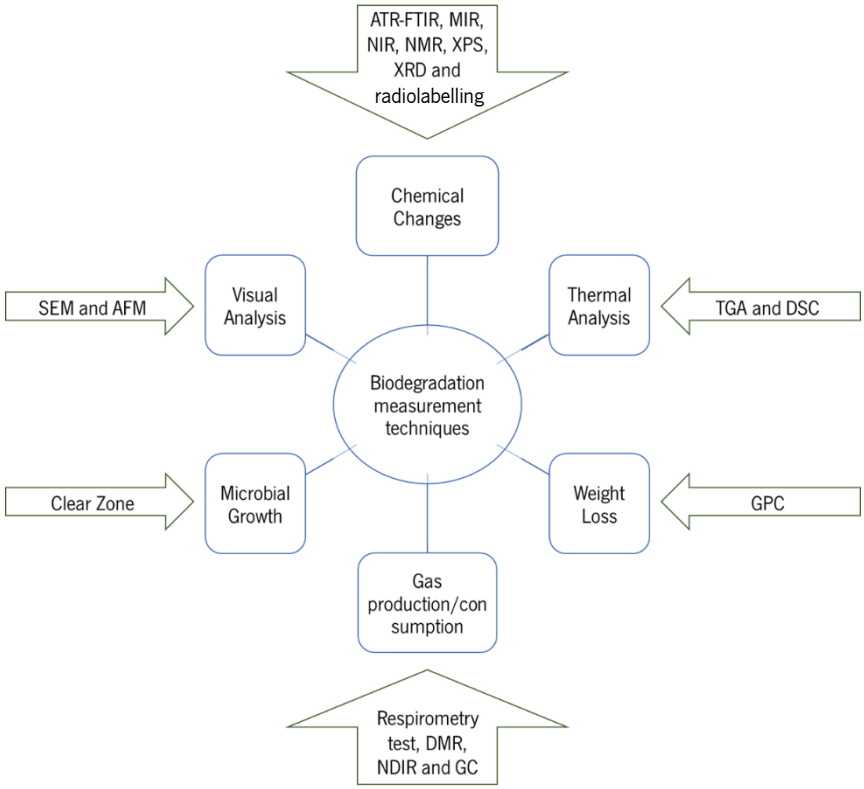

Figure 2.11 Analytical techniques to analyse and monitor plastic biodegradation. SEM- scanning electron microscopy, AFM- atomic force microscopy, TGA- thermogravimetric analysis, DSC- differential scanning calorimetry; GPC- Gel permeation chromatography, DMR- direct measurement respirometry, NDIR- nondispersive infrared sensor; GC- gas chromatography, ATR-FTIR - Attenuated total reflectance infrared spectrometry, MIR- mid-infrared, NIR- Near infrared and NMR- nuclear magnetic resonance, XPS- X-ray photoelectron spectroscopy and XRD- X-ray diffraction spectroscopy. Adapted from Atanasova et al. (2021).

2.7.1 Clear zone technique

This technique can be used to identify microorganisms capable of biodegrading a specific polymer. Environmental samples or even biofilms developed on the surface of the polymers, can be cultivated on

typical microbiological media containing the polymers as the unique carbon source (Volova et al., 2017). The polymers are dispersed in the agar as fine particles (micro or nanoparticles). Then if the microorganisms can produce extracellular enzymes capable of degrading the polymer into water-soluble components, clear zones (halo) are formed in the agar plate (Boyandin et al., 2012b; Schirmer et al., 1995). Other types of microorganisms (commensal organisms) may develop on the surface by using the intermediates of the polymer biodegradation such as oligomers, monomers, acetoacetate, and other products, however in this case no clear zones are formed (Volova et al., 2017). Charnock (2021) used this technique to isolate bacteria capable of degrading PHB, PHBV and PCL between other polymers from Norwegian water and soils.

2.7.2 Macro and micro visual analysis of the polymer surface

SEM is used to analyse the surface of a sample with a focused beam of electrons. The interactions between the electrons light and atoms of the surface produce several signals that are detected and create an image. These images provide information about the surface topography, morphology, and composition of the material (Choudhary and Choudhary, 2017). SEM is one of the most used techniques to study morphological alterations in polymer structures that were subjected to biodegradation. Usually, pre-exposed samples or controls are compared with the exposed ones. This method can be used to observe the effects of bacterial hydrolysis in polymer degradation (holes, cracks and erosion) or to evaluate the effect of factors such as temperature or pH on biodegradation assays through the surface analysis (Gómez and Michel, 2013; Kuntanoo and Promkotra, 2013; Luo and Netravali, 2003). These visual changes indicate microbial attack but not metabolic biodegradation (Gómez and Michel, 2013). SEM is often considered a valuable tool for studying the colonization and biodegradation of PHA films. For example, it was applied to evaluate the biodegradability of PHAs by soil microorganisms, since polymer deterioration generally occurs through surface erosion, due to microbial activity (Gonçalves et al., 2009; Lopez-Llorca et al., 1993; Tao et al., 2009).

Atomic force microscopy (AFM) is a kind of scanning probe microscopy, with a resolution on the order of a nanometre or lower. It has been used to visualize PHBV polymer surface morphology (macroscopy features) during degradation and study its roughness or colour changes, which is very useful for the characterization of surfaces (Arcos-Hernandez et al., 2012). TEM can also be used, to study enzymatic biodegradation and to analyse the surface of a polymer since it uses a beam of electrons that is transmitted through a sample to form an image (Nobes et al., 1996).

2.7.3 Spectroscopy

It is possible to identify the chemical changes in the polymer structure and thus biodegradation through spectrum alterations using spectroscopy methods (Tabasi and Ajji, 2015). The changes can indicate the formation of low molecular weight products originating from the polymer degradation.

Infrared spectroscopy measures the interaction of infrared radiation with polymers by absorption, emission, or reflection. The near-infrared (NIR) region is normally defined to range from 400 to 700 nm (visible range) to 2500 nm of the spectrum, being NIR spectroscopy mainly based on the absorption bands of OH, CH and NH bonds (Mulbry et al., 2012). The mid-infrared (MIR) spectroscopy (MIRS) uses the 2500 to 25000 nm range and includes several other bonds such as aromatic rings and carbon-related (Mulbry et al., 2012). Fourier-transform mid-infrared spectroscopy (FTIR) is a vibrational spectroscopy technique that measures the vibrational energy levels of molecules or groups of atoms in samples, especially solids exposed to a source of radiation, allowing the detection of the molecule or atom (Koenig, 2001). An FTIR spectrometer can collect data over a wide spectral range simultaneously. Typically, polymers present characteristic infrared absorption bands at given wavenumbers which can be correlated to crystallinity. The precise band locations diverge according to the chemical composition of the polymer. FTIR is used before and after biodegradation tests, because it gives insights into the chemical structure of the polymer, since the increase in the number of peaks, in relation to the initial polymer spectrum indicates an increase in simple bounds, corresponding to the breakdown of the complex polymer, into simpler molecules (Wei et al., 2015b; Weng et al., 2010; Wu, 2014). Attenuated total reflectance (ATR) is used with FTIR to measure surface properties of solid or thin polymer films, which allows following the changes in the structure of samples through the biodegradation process (Wei et al., 2015b). Its frequently used with several types of polymers including PHAs, after soil/compost incubations or with isolated species, revealing the disappearance or decrease in peaks demonstrating bond breakdown (Mousavioun et al., 2012; Shah et al., 2010; Tabasi and Ajji, 2015). The same strategy has been used with PBAT films incubated in soil (Han et al., 2021).

Nuclear magnetic resonance (NMR) spectroscopy is a physical observation in which nuclei (C, H, N, P, or O resonate at different energies) in a strong constant magnetic field are disturbed by a weak oscillating magnetic field and respond by creating an electromagnetic signal with a frequency distinctive of the magnetic field at the nucleus (Darbeau, 2006). NMR can be used to determine the monomeric composition and distribution of polymers and to see their evolution along the biodegradation process. Normally, two types of NMR techniques can be used ^{1}H-NMR and ^{13}C-NM, individually, or in combination to provide more information (Arcos-Hernandez et al., 2012; Wu, 2014). ^{1}H-NMR is more sensitive and

requires less analytical time. ^{13}C-NMR can take longer and is less sensitive. However, ^{13}C-NMR is superior in the analysis of macromolecules and long carbon chains of monomers. NMR quantitative estimation of PHA monomers was performed using the intensity ratio of the signals (Arcos-Hernandez et al., 2012). The application of ^{1}H-NMR in PBAT incubated in several compost environments demonstrated that the amorphous regions were more susceptible to hydrolysis and biodegradation than the aromatic domain (Kijchavengkul et al., 2010b). Mass spectroscopy (MS) is an analytical technique also used to identify products during the enzymatic degradation of polymers normally in combination with liquid or gas chromatography (GC) (Jia et al., 2021; Shah et al., 2013b). Witt et al., (2001) used GC-MS to test the PBAT degradation potential of a thermophilic strain *Thermomonospora fusca,* revealing that only the monomers were detected.

X-ray photoelectron spectroscopy (XPS) is a surface-sensitive quantitative spectroscopic method that has the capability of presenting the elements present in a film. (Corrêa et al., 2008). It can also reveal other elements and at what they are bonded to, for example, it identified the accumulation of biofilms on the surface of buried lignin/PHB samples (Mousavioun et al., 2012). The spectra are acquired by irradiating the material with a beam of X-rays and simultaneously determining the kinetic energy and number of electrons that leak from the top 0 to 10 nm of the material being examined. Polymer crystallinity can be evaluated using methods based on X-ray diffraction (XRD). XRD irradiates a polymer with incident X-rays and then measures the intensities and scattering angles of the X-rays that are reflected from the material. XRD is a non-destructive technique that allows the measurement of absolute crystallinity and atomic structures such as chemical bonds (Boyandin et al., 2013; Ong and Sudesh, 2016). This technique demonstrated that isolated *Bacillus* species degraded the amorphous phase of PBAT films (Zhang et al., 2021).

Radiolabelling can also be used to assess the degree of biodegradation using radiolabelled carbon, The carbon atoms in the polymer backbone are labelled with carbon isotopes ^{13}C (stable) (Zumstein et al., 2018). Then the carbon is tracked into CO_2 and biomass. This technique was successfully applied with PBAT films incubated in soil (Zumstein et al., 2018). This method is very precise, without any type of interference, however, is relatively difficult to use, requires skilled laboratories and is expensive (Zumstein et al., 2018).

2.7.4 Thermal analysis

Thermal analysis is the evaluation of the material behavior in response to temperature. Differential scanning calorimetry (DSC) measures the heat flow to and from a sample as a function of temperature

as the sample is heated, cooled, or held at a constant temperature. DSC is the favourite method to be used because it provides qualitative and quantitative thermal information (Schick, 2009). Glass transition temperature (T_g) and melting temperature (T_m) are commonly studied, and also heat capacities and crystallinity (Arcos-Hernandez et al., 2012; Shrivastav et al., 2011). The thermo-degradation temperature of polymers is obtained using thermogravimetric analysis (TGA), a technique where a sample is heated in a controlled atmosphere at a well-defined rate while sample mass loss is measured. The decrease in the polymer thermal stability after biodegradation is an indicator of degradation (Batista et al., 2010). Several studies use these techniques to evaluate thermal transitions in polymer samples including with PHAs and PBAT since due to biodegradation, the stability of the polymers decreases, causing changes in crystallinity (usually increases) and phase transition temperatures (Kijchavengkul et al., 2010b; Mousavioun et al., 2012).

2.7.5 Chromatography

Gel permeation chromatography (GPC) is used to measure the polymer molecular weight, so it can be used to detect weight reduction during biodegradation (Shah et al., 2013b). The molecular weight reduction and distribution indicate the occurrence of a degradation process. The reduction of the molecular weight has been demonstrated with PBAT films after biodegradation and also with other polymers such as PLA and PHAs (Fu et al., 2020; Sin et al., 2010). However, this analysis only considers bulk polymer, not analysing the changes on the surface, and the biofilm development can also interfere with the measurement. When accompanied by other data, such as mineralization, the GPC analysis presents more insights into understanding of the biodegradation process (Kasuya et al., 2009).

High-performance liquid chromatography (HPLC) is commonly used for the qualitative and quantitative analysis of soluble compounds resulting from enzymatic activity (Nakajima-Kambe et al., 2009a). The polymers are dissolved in an organic solvent and injected into an HPLC column with a cross-linked gel that causes the molecules separation according to the size (Nakajima-Kambe et al., 2009a). Qualitative data on the branching of the sample and the identification of the composition distribution of it can be acquired (Oda et al., 1995). For example, an HPLC analysis demonstrated that *Leptothrix* sp. strain TB-71 degrades PBAT into monomers (Nakajima-Kambe et al., 2009a).

2.7.6 Weight loss

Weight loss indicates the mass loss determined from the samples recovered during the degradation test when compared to the initial mass. Although some works use weight loss to assess biodegradation in

soil, sometimes it is difficult to adequately clean the samples following soil burial and obtain the exact value (Wei et al., 2015b; Wu et al., 2017; Zaidi et al., 2019). It is mostly employed to designate the degradation occurring on the surface. Some authors even indicate that it is impossible to use weight loss in later stages of the tests, due to a high degradation level of the material, hindering a proper weight evaluation. Biodegradation implies that the polymers are broken down into simple molecular units such as CO_2 and water (Müller, 2005). Weight loss is not necessarily considered an indicator of biodegradability in the sense of most definitions, so the conclusions obtained from weight loss can be related to biodegradation, but they should not be used alone as a biodegradation assessment (Müller, 2005). This type of method has been used with several polymers such as PBAT and PHAs to study the degradation capability of isolates and communities or in simulation and field works and can be useful when applied in parallel with several other methodologies such as XRD, FTIR and BOD (Iggui et al., 2015; Soulenthone et al., 2020; Zhang et al., 2021).

2.7.7 Respirometric tests for CO_2 evolution and biochemical O_2 demand

In aerobic conditions, microorganisms use oxygen and produce carbon dioxide, because of the oxidation of the carbon existing in the polymer. Methodologies that can track the consumption of O_2 or the formation of CO_2 are extremely useful and recommended by the standard methodologies (ASTM D5988-18 (2018), ISO 17566 (2019)). The evolved CO_2 can be trapped in a basic solution (sodium hydroxide or barium hydroxide) and then quantified by the titration method with hydrochloric acid, in a cumulative measurement respirometry (Briassoulis et al., 2020). Arcos-Hernandez et al., (2012) used this approach to test the biodegradation of PHBV in active sludge and Saadi et al., (2013) did the same with PBAT incubated in soil and compost. This methodology can be executed in closed systems, where no aeration occurs, or in dynamic assays, flows through tests (Briassoulis et al., 2020). The BOD is normally accomplished in closed reactors, with any type of polymer such as PHBV nanocomposites (Iggui et al., 2015). The evolved CO_2 can be quantified using an inline non-dispersive infrared gas analyser or GC (direct measurement respirometry) (Castro-Aguirre et al., 2017). The evolved CO_2 may also be trapped in absorption columns, and the increased weight is employed to quantify the amount of CO_2 (gravimetric measurement respirometry) (Kale et al., 2007).

In summary, several methods are frequently used simultaneously to determine biodegradation. The change in weight loss, molecular weight, and surface analysis are widely used, but they don't clearly indicate biodegradation. The CO_2, O_2 analysis and radiolabelling can demonstrate the total assessment of the breakdown of the polymer into biomass. The respirometry methods are recommended by the standard

guidelines since they determine the mineralization value, nevertheless radiolabelling presents the actual assimilation of the polymer carbon into the microbial biomass.

2.8 Soil standard testing methods for plastic biodegradation

Several organizations, such as the ASTM and ISO have started rigorous programs to create new standardized tests for studying the biodegradability of solid polymers (Itävaara and Vikman, 1995). Concerning aerobic biodegradation of plastics in soil, the most appropriate based standard testing methods are ASTM D5988 – 18 (2018), ISO 17556 (2019), and the French and Italian norms NF U52-001 (2005) and UNI 11462 (2012), respectively as indicated in Table 2.5 (Briassoulis and Mistriotis, 2018).

Table 2.5 Overview of soil standard testing methods for determining biodegradability of plastic materials

American Society for Testing and Materials	
ASTM D5988-18	Determination of aerobic biodegradation of plastic materials in soil
International Organization for Standardization	
ISO 17556 -2019	Plastics -Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved
French and Italian Normalisation Organisations (ANFNOR, UNI)	
NF U52-001:2005	Biodegradable materials for use in agriculture and horticulture - Mulching products - Requirements and test methods
UNI 11462 - 2012	Plastic materials biodegradable in soil - Types, requirements, and test methods

The NF U52-001 indicates an equivalent biodegradation method in soil to the ASTM D5988-18 (2018) (Šerá et al., 2020). However, this standard was superseded by EN 17033 (2018) for "Plastics - Biodegradable mulch films for use in agriculture and horticulture - Requirements and test methods". This standard was created using the methodology of ISO 17556 (Šerá et al., 2020). All these standards are not exactly equal, which may lead to results that depend on the standard test method used. The main differences among these standards are associated with the carbon:nitrogen ratio (C:N) concerning the mass of carbon that is in the sample, soil medium, soil pH, test sample and water content of the soil as explained in Table 2.6.

Table 2.6 Main differences between soil standard testing methods, in terms of soil medium, test sample, soil pH, C:N ratio and water content

Soil medium
ASTM D5988: Laboratory mixture of equal parts of natural and fertile soil samples collected from the surface layers of fields and forests obtained from at least 3 diverse locations or a mixture of natural soil and mature compost;
ISO 17566: Use of a "standard soil" as an alternative to natural soil that constitutes of industrial quartz sand, clay, natural soil, and mature compost;
NF U52-001: Natural soil with organic Carbon < 2 %;
Test sample
ASTM D5988: Sample quantity of 200-1000 mg carbon for 500 g soil;
ISO 17566: Sample quantity of 100-300 mg to 100-300 g of soil; test samples may be reduced in size using cryogenic milling. When the CO_2 production is measured, higher test material quantities can be used;
NF U52-001: Sample quantity of 200-1000 mg of organic carbon in 500g of soil substrate; test samples film must be cut to pieces 1-2 cm or powder;
Soil pH
ASTM D5988: Soil pH should be 6-8;
ISO 17566: Soil pH should be 6-8; pH can be adjusted;
NF U52-001: Soil pH should be 6-8, pH can be adjusted;
C:N Ratio
ASTM D5988: C:N ratio adjusted to a value between 10:1 and 20:1 by weight to the added carbon in the test specimen (note defined explicitly; presumably for the C:N of test sample; needs clarification though);
ISO 17566: The ratio C:N is at least 40:1 for the sample organic C to the soil N;
NF U52-001: C:N ratio adjusted to a value between 10:1 and 20:1 in relation to the mass of carbon contained in the sample and total N contained in the soil. The same amount of nitrogen is added in the reactors containing the soil blanks;
Water content of the soil
ASTM D5988: Water content 80-100 % of moisture-holding capacity (MHC) (by D425) or 50 to 70 % (by D2980);
ISO 17566: Water content 40-60 % of the total water holding capacity of the soil; specific salts may be added in the soil preferably when adjusting the water content;
NF U52-001: Water content 80 % of the total water holding capacity of the soil;

The CO_2 produced is generally used for quantifying biodegradation. The two most used systems are the ASTM D5988 and the ISO 17566. The ISO 17566 uses a method where the CO_2 production is measured in an aerated system with continuous CO_2-free air (Briassoulis et al., 2020). Although different from the ASTM D5988 system these 2 standard methods refer each other and even indicate that the test can be done alternatively by any of the 2 systems. Sometimes ISO 17566 is implemented, and its specifications are used (i.e. soil medium, water content), but with the ASTM system (Briassoulis et al., 2020). This occurs because, in logistics terms, it is easier to implement compared to the ISO system since the latter

requires besides the air-flow system for measuring carbon dioxide, much more space and resources (Briassoulis et al., 2020).

The most significant weakness of the existing standards for testing the biodegradation of plastics in soil is their weak reproducibility (Briassoulis and Mistriotis, 2018). Several factors including the soil type and biodiversity, the conditions such as temperature, water content or pH, and the measuring method may affect the reproducibility of the results (Briassoulis and Mistriotis, 2018). For example, if plastic is the only carbon source existing for microorganism growth the test may present a higher rate of degradation compared to an environment with other carbon sources that may be better for the microorganisms. The tested material may also be biodegradable in testing conditions at a specific rate however, because the properties in real environments vary extensively (e.g., microorganism and environmental conditions), the results may be not representative of those environments. The NF U52-001 and UNI 11462 set a time frame and pass levels for biodegradation of biodegradable materials used in agriculture and horticulture. The European Standard specification EN 17033 (2018) set some standards regarding the biodegradation of plastic mulch films, including minimum biodegradation of 90 % under aerobic conditions in natural agricultural or forest topsoil within a maximum period of 2 years.

2.9 Genomic markers for taxonomic identification of microorganisms

The microbial communities existing in different environments such as soil, can be extremely diverse. This diversity greatly increases the chances of identifying microorganisms with fundamental functions involved in polymer biodegradation. Strategies to identify such microorganisms include culture-dependent methods that present several drawbacks including, that most microorganisms are not cultivable or are very difficult to cultivate, do not grow on selective media and some, only grow in communities, and their function can differ from what they do when growing in pure cultures (Liu et al., 2022a).

Several techniques have been applied in the past to analyse microbial diversity, such as DGGE, terminal restriction fragment length polymorphism (T-RFLP) and fluorescent in situ hybridization (FISH) (Yang et al., 2016). Nevertheless, modern metataxonomics methods involving next-generation sequencing (NGS), have enabled and increased knowledge regarding microorganisms communities (Yang et al., 2016). These methods depend mostly on the sequencing of taxonomic biomarkers, normally the genes encoding the ribosomal RNA (rRNA). The sequencing of the rRNA gene is extremely effective for the identification of microorganisms. The rRNA is present in all organisms and has a permanent functionality and a

dominant concentration in the cells since is it necessary for the transduction of new proteins (Boughner and Singh, 2016). It is composed of highly conserved regions, similar to prokaryotes and eukaryotes that can be used as targets for PCR primers (Yang et al., 2016). It has 9 variable regions, which allow the identification and taxonomical differentiation (Yang et al., 2016). 16S and 18S rRNA genes, encode the 30s subunit of the prokaryotic ribosome, and the 40s subunit of the eukaryotic ribosome, respectively, and are widely used for the identification of prokaryotic and eukaryotic microorganisms in microbial communities (Wu et al., 2015; Yang et al., 2016). The Internal Transcribed Spacer (ITS) region removed in the posttranscriptional process of nuclear rRNA cistron, has been extensively considered as a universal fungi marker, allowing an efficient identification of the broadest range of fungi (Schoch et al., 2012). The ITS is more variable than 18S and can be more fitting as a genetic marker for determining intraspecific genetic diversity (Schoch et al., 2012).

2.10 Methodologies for taxonomic identification

2.10.1 Illumina sequencing

Illumina sequencing methods (MiSeq, HiSeq, and NextSeq) are the most widely used NGS platforms (Phadke et al., 2017). These sequencing technologies allow simultaneously the identification of the microorganisms and the determination of their relative abundance in the communities (Phadke et al., 2017). Normally it is necessary to isolate DNA from the sample and to separate the genetic material from all the other constituents of the cell. Depending on the sample origin some contaminants, and inhibitors may be present, and the extraction protocol should be adjusted. Then the rRNA genes of interest may be amplified by PCR using primers. Using Illumina sequencing, after an initial PCR amplification targeting the gene of interest, another PCR is performed using primers with adapters which allow the attachment of Illumina sequencing adaptors and indices (also called barcodes) to the samples in study (Phadke et al., 2017). The gene sequences obtained can be then compared with other sequences in databases (i.e., NCBI) to perform the taxonomic identification. The degree of similarity (in percentage) between the sequences, determines the taxonomic assignment of the microorganisms. This kind of approach has been applied for example to study the biodegradation of plastics. The use of Illumina sequencing with soil samples with PBAT and PHAs polymers gave insights into the impact of the biodegradation on the microbial communities of the surrounding sediments, and shed some light on the potential microorganisms capable of using the polymers as carbon sources (Vannini et al., 2021; Zhang et al., 2022).

2.10.2 Sanger sequencing

An old technique to identify isolated microorganisms is the complete 16S rRNA or 18S rRNA sequencing with Sanger technology, especially when high throughput is not necessary. This is important because a nearly complete gene sequence allows a more reliable taxonomic assignment.

The principle of the technique is the following: It uses primers, for example, specific for 16S or 18S rRNA or another region that is a biomarker. After denaturation of the DNA, and the primers annealing the elongation occurs with deoxynucleotide triphosphates (arginine, cytosine, tyrosine, and guanine) and dideoxynucleotide triphosphates (ddNTPs) (which miss the 3´ hydroxyl group needed for the extension of a DNA polynucleotide chain) (Gomes and Korf, 2018). This originates strands of each possible length when the ddNTPs are randomly included which terminates the chain elongation (Solomon, 2018). The ddNTPs have a fluorescent marker with a colour for each type, that will fluoresce in the chain based on the connected nucleotide (Gomes and Korf, 2018). Then the signal produced by each excited nucleotide corresponds to a certain base, creating a chromatograph that displays the fluorescent peak of each labelled fragment (Solomon, 2018). The sequence obtained is compared with others using databases to identify the microorganism based on the similarity. Is very useful after the isolation of microorganisms capable of degrading polymers such as PHAs in specific media (Boyandin et al., 2012a).

2.11 REFERENCES

Aarthy, M., Puhazhselvan, P., Aparna, R., George, A. S., Gowthaman, M. K., Ayyadurai, N., … Kamini, N. R. (2018). Growth associated degradation of aliphatic-aromatic copolyesters by Cryptococcus sp. MTCC 5455. *Polymer Degradation and Stability*, 152, 20–28. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2018.03.021

Adediran, A. A., Akinwande, A. A., Balogun, O. A., Bello, O. S., Akinbowale, M. K., Adesina, O. S., & Ojo, A. A. (2022). Mechanical and optimization studies of polypropylene hybrid biocomposites. *Scientific Reports*, 12(1), 2468. Retrieved from https://doi.org/10.1038/s41598-022-06304-6

Ajellal, N., Thomas, C. M., & Carpentier, J.-F. (2009). Functional syndiotactic poly(β-hydroxyalkanoate)s via stereoselective ring-opening copolymerization of rac-β-butyrolactone and rac-allyl-β-butyrolactone. *Journal of Polymer Science Part A: Polymer Chemistry*, 47(12), 3177–3189. Retrieved from https://doi.org/https://doi.org/10.1002/pola.23418

Ajmal, A. W., Masood, F., & Yasin, T. (2018). Influence of sepiolite on thermal, mechanical and biodegradation properties of poly-3-hydroxybutyrate-co-3-hydroxyvalerate nanocomposites. *Applied Clay Science*, 156, 11–19. Retrieved from https://doi.org/https://doi.org/10.1016/j.clay.2018.01.010

Akmal, D., Azizan, M. N., & Majid, M. I. A. (2003). Biodegradation of microbial polyesters P(3HB) and P(3HB-co-3HV) under the tropical climate environment. *Polymer Degradation and Stability*, 80(3), 513–518. Retrieved from https://doi.org/https://doi.org/10.1016/S0141-3910(03)00034-X

Al-Itry, R., Lamnawar, K., & Maazouz, A. (2012). Improvement of thermal stability, rheological and mechanical properties of PLA, PBAT and their blends by reactive extrusion with functionalized epoxy. *Polymer Degradation and Stability*, 97(10), 1898–1914. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.06.028

Ali, S. S., Elsamahy, T., Al-Tohamy, R., Zhu, D., Mahmoud, Y. A. G., Koutra, E., ... Sun, J. (2021). Plastic wastes biodegradation: Mechanisms, challenges and future prospects. *Science of the Total Environment*, 780, 146590. Retrieved from https://doi.org/10.1016/j.scitotenv.2021.146590

Altaee, N., El-Hiti, G. A., Fahdil, A., Sudesh, K., & Yousif, E. (2016). Biodegradation of different formulations of polyhydroxybutyrate films in soil. *SpringerPlus*, 5(1), 762. Retrieved from https://doi.org/10.1186/s40064-016-2480-2

Arcos-Hernandez, M. V, Laycock, B., Pratt, S., Donose, B. C., Nikolić, M. A. L., Luckman, P., ... Lant, P. A. (2012). Biodegradation in a soil environment of activated sludge derived polyhydroxyalkanoate (PHBV). *Polymer Degradation and Stability*, 97(11), 2301–2312. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.07.035

Arrieta, M. P., López, J., Ferrándiz, S., & Peltzer, M. A. (2013). Characterization of PLA-limonene blends for food packaging applications. *Polymer Testing*, 32(4), 760–768. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2013.03.016

Ashby, R., & Solaiman, D. (2008). Poly(hydroxyalkanoate) Biosynthesis from Crude Alaskan Pollock (Theragra chalcogramma) Oil. *Journal of Polymers and the Environment*, 16(4), 221–229. Retrieved from https://doi.org/10.1007/s10924-008-0108-5

Aso, T., Koda, K., Kubo, S., Yamada, T., Nakajima, I., & Uraki, Y. (2013). Preparation of Novel Lignin-Based Cement Dispersants from Isolated Lignins. *Journal of Wood Chemistry and Technology*, 33(4), 286–298. Retrieved from https://doi.org/10.1080/02773813.2013.794841

ASTM D5988-18. (2018). Standard Test Method for Determining Aerobic Biodegradation in Soil of Plastic Materials. *ASTM International*

ASTM D883-18. (2018). Standard Terminology Relating to plastics. *ASTM International*

Atanasova, N., Stoitsova, S., Paunova-Krasteva, T., & Kambourova, M. (2021). Plastic Degradation by Extremophilic Bacteria. *International Journal of Molecular Sciences*, 22(11). Retrieved from https://doi.org/10.3390/ijms22115610

Atiwesh, G., Mikhael, A., Parrish, C. C., Banoub, J., & Le, T.-A. T. (2021). Environmental impact of bioplastic use: A review. *Heliyon*, 7(9), e07918. Retrieved from https://doi.org/https://doi.org/10.1016/j.heliyon.2021.e07918

Avella, M., Rota, G. La, Martuscelli, E., Raimo, M., Sadocco, P., Elegir, G., & Riva, R. (2000). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and wheat straw fibre composites: thermal, mechanical properties and biodegradation behaviour. *Journal of Materials Science*, 35(4), 829–836. Retrieved from https://doi.org/10.1023/A:1004773603516

Ayyoob, M., Lee, D. H., Kim, J. H., Nam, S. W., & Kim, Y. J. (2017). Synthesis of poly(glycolic acids) via solution polycondensation and investigation of their thermal degradation behaviors. *Fibers and Polymers*, 18(3), 407–415. Retrieved from https://doi.org/10.1007/s12221-017-6889-1

Baghi, F., Gharsallaoui, A., Dumas, E., & Ghnimi, S. (2022). Advancements in Biodegradable Active Films for Food Packaging: Effects of Nano/Microcapsule Incorporation. *Foods*, 11(5). Retrieved from https://doi.org/10.3390/foods11050760

Bai, X., & Kim, K. H. (2016). Biofuels and chemicals from lignin based on pyrolysis. *Production of Biofuels and Chemicals from Lignin*, 263–287. Retrieved from https://doi.org/10.1007/978-981-10-1965-4_9

Baidurah, S., Murugan, P., Sen, K. Y., Furuyama, Y., Nonome, M., Sudesh, K., & Ishida, Y. (2019). Evaluation of soil burial biodegradation behavior of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) on

the basis of change in copolymer composition monitored by thermally assisted hydrolysis and methylation-gas chromatography. *Journal of Analytical and Applied Pyrolysis*, 137, 146–150. Retrieved from https://doi.org/https://doi.org/10.1016/j.jaap.2018.11.020

Barkoula, N. M., Garkhail, S. K., & Peijs, T. (2010). Biodegradable composites based on flax/polyhydroxybutyrate and its copolymer with hydroxyvalerate. *Industrial Crops and Products*, 31(1), 34–42. Retrieved from https://doi.org/https://doi.org/10.1016/j.indcrop.2009.08.005

Barnes, D., Galgani, F., Thompson, R., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 364, 1985–1998. Retrieved from https://doi.org/10.1098/rstb.2008.0205

Barragán, D. H., Pelacho, A. M., & Martin-Closas, L. (2016). Degradation of agricultural biodegradable plastics in the soil under laboratory conditions. *Soil Research*, 54(2), 216–224. Retrieved from https://doi.org/10.1071/SR15034

Bassas Galià, M. (2010). Isolation and Analysis of Storage Compounds BT - Handbook of Hydrocarbon and Lipid Microbiology. In K. N. Timmis (Ed.) (pp. 3725–3741). Berlin, Heidelberg: Springer Berlin Heidelberg. Retrieved from https://doi.org/10.1007/978-3-540-77587-4_292

Bastioli, C. (2005). Handbook of biodegradable polymers. Shawbury, Shrewsbury, Shropshire, U.K.: Rapra Technology. Retrieved from https://doi.org/LK - https://worldcat.org/title/246629782

Batista, K., Silva, D. A. K., Coelho, L., Pezzin, S., & Pezzin, A. (2010). Soil Biodegradation of PHBV/Peach Palm Particles Biocomposites. *Journal of Polymers and the Environment*, 18, 346–354. Retrieved from https://doi.org/10.1007/s10924-010-0238-4

Battegazzore, D., Frache, A., Abt, T., & Maspoch, M. L. (2018). Epoxy coupling agent for PLA and PHB copolymer-based cotton fabric bio-composites. *Composites Part B: Engineering*, 148, 188–197. Retrieved from https://doi.org/https://doi.org/10.1016/j.compositesb.2018.04.055

Beckers, S., Peil, S., & Wurm, F. R. (2020). Pesticide-Loaded Nanocarriers from Lignin Sulfonates—A Promising Tool for Sustainable Plant Protection. *ACS Sustainable Chemistry & Engineering*, 8(50), 18468–18475. Retrieved from https://doi.org/10.1021/acssuschemeng.0c05897

Berger, E., Ramsay, B. A., Ramsay, J. A., Chavarie, C., & Braunegg, G. (1989). PHB recovery by hypochlorite digestion of non-PHB biomass. *Biotechnology Techniques*, 3(4), 227–232. Retrieved from https://doi.org/10.1007/BF01876053

Bhati, R., & Mallick, N. (2012). Production and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) co-polymer by a N2-fixing cyanobacterium, Nostoc muscorum Agardh. *Journal of Chemical Technology & Biotechnology*, 87(4), 505–512. Retrieved from https://doi.org/https://doi.org/10.1002/jctb.2737

Bher, A., Mayekar, P. C., Auras, R. A., & Schvezov, C. E. (2022). Biodegradation of Biodegradable Polymers in Mesophilic Aerobic Environments. *International Journal of Molecular Sciences*, 23(20). Retrieved from https://doi.org/10.3390/ijms232012165

Bianco, A., Calderone, M., & Cacciotti, I. (2013). Electrospun PHBV/PEO co-solution blends: microstructure, thermal and mechanical properties. *Materials Science & Engineering. C, Materials for Biological Applications*, 33(3), 1067–1077. Retrieved from https://doi.org/10.1016/j.msec.2012.11.030

Bispo, V. M., Mansur, A. A. P., Barbosa-Stancioli, E. F., & Mansur, H. S. (2010). Biocompatibility of nanostructured chitosan/poly (vinyl alcohol) blends chemically crosslinked with genipin for biomedical applications. *Journal of Biomedical Nanotechnology*, 6(2), 166–175. Retrieved from https://doi.org/10.1166/jbn.2010.1110

Biundo, A., Hromic, A., Pavkov-keller, T., Gruber, K., Quartinello, F., Haernvall, K., ... Guebitz, G. M. (2016). Characterization of a poly (butylene adipate- co -terephthalate) - hydrolyzing lipase from Pelosinus fermentans. *Applied Microbiology and Biotechnology,* 100, 1753–1764. Retrieved from

https://doi.org/10.1007/s00253-015-7031-1

Boughner, L. A., & Singh, P. (2016). Microbial Ecology: Where are we now? *Postdoc Journal : A Journal of Postdoctoral Research and Postdoctoral Affairs*, 4(11), 3–17. Retrieved from https://doi.org/10.14304/SURYA.JPR.V4N11.2

Boyandin, A N, Prudnikova, S. V, Filipenko, M. L., Khrapov, E. A., Vasil'ev, A. D., & Volova, T. G. (2012a). Biodegradation of polyhydroxyalkanoates by soil microbial communities of different structures and detection of PHA degrading microorganisms. *Applied Biochemistry and Microbiology*, 48(1), 28–36. Retrieved from https://doi.org/10.1134/S0003683812010024

Boyandin, Anatoly N, Prudnikova, S. V, Karpov, V. A., Ivonin, V. N., Đỗ, N. L., Nguyễn, T. H., ... Gitelson, I. I. (2013). Microbial degradation of polyhydroxyalkanoates in tropical soils. *International Biodeterioration & Biodegradation*, 83, 77–84. Retrieved from https://doi.org/https://doi.org/10.1016/j.ibiod.2013.04.014

Boyandin, Anatoly N, Rudnev, V. P., Ivonin, V. N., Prudnikova, S. V, Korobikhina, K. I., Filipenko, M. L., ... Sinskey, A. J. (2012b). Biodegradation of Polyhydroxyalkanoate Films in Natural Environments. *Macromolecular Symposia*, 320(1), 38–42. Retrieved from https://doi.org/10.1002/masy.201251004

Briassoulis, D, & Mistriotis, A. (2018). Key parameters in testing biodegradation of bio-based materials in soil. *Chemosphere*, 207, 18–26. Retrieved from https://doi.org/10.1016/j.chemosphere.2018.05.024

Briassoulis, Demetres, Mistriotis, A., Mortier, N., & Tosin, M. (2020). A horizontal test method for biodegradation in soil of bio-based and conventional plastics and lubricants. *Journal of Cleaner Production*, 242, 118392. Retrieved from https://doi.org/https://doi.org/10.1016/j.jclepro.2019.118392

Bucci, D. Z., Tavares, L. B. B., & Sell, I. (2005). PHB packaging for the storage of food products. *Polymer Testing*, 24(5), 564–571. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2005.02.008

Buranov, A. U., & Mazza, G. (2008). Lignin in straw of herbaceous crops. *Industrial Crops and Products*, 28(3), 237–259. Retrieved from https://doi.org/https://doi.org/10.1016/j.indcrop.2008.03.008

Byun, Y., & Kim, Y. T. (2014). Chapter 15 - Utilization of Bioplastics for Food Packaging Industry. In J. H. B. T.-I. in F. P. (Second E. Han (Ed.), *Food Science and Technology* (pp. 369–390). San Diego: Academic Press. Retrieved from https://doi.org/https://doi.org/10.1016/B978-0-12-394601-0.00015-1

Carofiglio, V. E., Stufano, P., Cancelli, N., De Benedictis, V. M., Centrone, D., Benedetto, E. De, ... Demitri, C. (2017). Novel PHB/Olive mill wastewater residue composite based film: Thermal, mechanical and degradation properties. *Journal of Environmental Chemical Engineering*, 5(6), 6001–6007. Retrieved from https://doi.org/https://doi.org/10.1016/j.jece.2017.11.013

Casarin, S., Malmonge, S., & Rosário, F. (2012). Biodegradable PHB/Copolyester Blends - Biodegradation in Soil. *Polímeros*, 23, 115–122. Retrieved from https://doi.org/10.1590/S0104-14282013005000003

Casarin, S., Rodrigues, C., Júnior, O., Rosário, F., & Agnelli, J. (2017). Biodegradation in Soil of the PHB/Wood Flour (80/20) and PHB/Sisal Fiber (80/20) Tubes. *Materials Research*, 20(S2), 47–50. Retrieved from https://doi.org/10.1590/1980-5373-mr-2016-0904

Castro-Aguirre, E., Auras, R., Selke, S., Rubino, M., & Marsh, T. (2017). Insights on the aerobic biodegradation of polymers by analysis of evolved carbon dioxide in simulated composting conditions. *Polymer Degradation and Stability*, 137, 251–271. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.017

Chan, C. M., Vandi, L.-J., Pratt, S., Halley, P., Richardson, D., Werker, A., & Laycock, B. (2019). Insights

into the biodegradation of PHA / wood composites: Micro- and macroscopic changes. *Sustainable Materials and Technologies*, 21, e00099. Retrieved from https://doi.org/https://doi.org/10.1016/j.susmat.2019.e00099

Chanasit, W., Martla, M., & Umsakul, K. (2023). Synthesis and biodegradation of polymer blends of poly(3-hydroxybutyrate) and natural rubber. *IOP Conference Series: Earth and Environmental Science*, 1139(1), 12006. Retrieved from https://doi.org/10.1088/1755-1315/1139/1/012006

Charnock, C. (2021). Norwegian soils and waters contain mesophilic, plastic-degrading bacteria. *Microorganisms*, 9(1), 1–18. Retrieved from https://doi.org/10.3390/microorganisms9010094

Chathalingath, N., Kingsly, J. S., & Gunasekar, A. (2023). Biosynthesis and biodegradation of poly(3-hydroxybutyrate) from Priestia flexa; A promising mangrove halophyte towards the development of sustainable eco-friendly bioplastics. *Microbiological Research*, 267, 127270. Retrieved from https://doi.org/https://doi.org/10.1016/j.micres.2022.127270

Chaudhry, W. N., Jamil, N., Ali, I., Ayaz, M. H., & Hasnain, S. (2011). Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. *Annals of Microbiology*, 61(3), 623–629. Retrieved from https://doi.org/10.1007/s13213-010-0181-6

Chen, G.-Q., & Wu, Q. (2005). The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials*, 26(33), 6565–6578. Retrieved from https://doi.org/https://doi.org/10.1016/j.biomaterials.2005.04.036

Cheng, K.-C., Catchmark, J. M., & Demirci, A. (2009). Effect of different additives on bacterial cellulose production by Acetobacter xylinum and analysis of material property. *Cellulose*, 16(6), 1033–1045. Retrieved from https://doi.org/10.1007/s10570-009-9346-5

Choi, G. G., Kim, H. W., & Rhee, Y. H. (2004). Enzymatic and non-enzymatic degradation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters produced by Alcaligenes sp. MT-16. *Journal of Microbiology (Seoul, Korea)*, 42(4), 346–352. Retrieved from https://pubmed.ncbi.nlm.nih.gov/15650693/

Choudhary, O. P., & Choudhary, P. (2017). Scanning Electron Microscope: Advantages and Disadvantages in Imaging Components. *International Journal of Current Microbiology and Applied Sciences*, 6, 1877–1882. Retrieved from https://doi.org/10.20546/ijcmas.2017.605.207

Chrissafis, K., Antoniadis, G., Paraskevopoulos, K. M., Vassiliou, A., & Bikiaris, D. N. (2007). Comparative study of the effect of different nanoparticles on the mechanical properties and thermal degradation mechanism of in situ prepared poly(ε-caprolactone) nanocomposites. *Composites Science and Technology*, 67(10), 2165–2174. Retrieved from https://doi.org/https://doi.org/10.1016/j.compscitech.2006.10.027

Colin, R., & BjØrn, K. (2006). *Basic biotechnology: Third edition*. *Basic Biotechnology: Third Edition*. Retrieved from https://doi.org/10.1017/CBO9780511802409

Conn, R. E., Kolstad, J. J., Borzelleca, J. F., Dixler, D. S., Filer Jr., L. J., Ladu Jr., B. N., & Pariza, M. W. (1995). Safety assessment of polylactide (PLA) for use as a food-contact polymer. *Food and Chemical Toxicology*, 33(4), 273–283. Retrieved from https://doi.org/10.1016/0278-6915(94)00145-E

Conte, E., Catara, V., Greco, S., Russo, M., Alicata, R., Strano, L., ... Catara, A. (2006). Regulation of polyhydroxyalkanoate synthases (phaC1 and phaC2) gene expression in Pseudomonas corrugata. *Applied Microbiology and Biotechnology*, 72(5), 1054–1062. Retrieved from https://doi.org/10.1007/s00253-006-0373-y

Corrêa, M. C. S., Rezende, M. L., Rosa, D. S., Agnelli, J. A. M., & Nascente, P. A. P. (2008). Surface composition and morphology of poly(3-hydroxybutyrate) exposed to biodegradation. *Polymer Testing*, 27(4), 447–452. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2008.01.007

Corti, A., Solaro, R., & Chiellini, E. (2002). Biodegradation of poly(vinyl alcohol) in selected mixed microbial culture and relevant culture filtrate. *Polymer Degradation and Stability*, 75(3), 447–458. Retrieved from https://doi.org/https://doi.org/10.1016/S0141-3910(01)00247-6

Cruz, M. V, Freitas, F., Paiva, A., Mano, F., Dionísio, M., Ramos, A. M., & Reis, M. A. M. (2016). Valorization of fatty acids-containing wastes and byproducts into short- and medium-chain length polyhydroxyalkanoates. *New Biotechnology*, 33(1), 206–215. Retrieved from https://doi.org/https://doi.org/10.1016/j.nbt.2015.05.005

da Silva Moura, A., Demori, R., Leão, R. M., Crescente Frankenberg, C. L., & Campomanes Santana, R. M. (2019). The influence of the coconut fiber treated as reinforcement in PHB (polyhydroxybutyrate) composites. *Materials Today Communications*, 18, 191–198. Retrieved from https://doi.org/https://doi.org/10.1016/j.mtcomm.2018.12.006

Daisuke, K., Toshihiro, F., Tomokuni, A., Kohei, M., Yoshihiro, K., Masao, F., & Eiji, M. (2009). Uncovering the Protocatechuate 2,3-Cleavage Pathway Genes. *Journal of Bacteriology*, 191(21), 6758–6768. Retrieved from https://doi.org/10.1128/jb.00840-09

Darbeau, R. (2006). Nuclear Magnetic Resonance (NMR) Spectroscopy: A Review and a Look at Its Use as a Probative Tool in Deamination Chemistry. *Applied Spectroscopy Reviews - Appl Spetrosc Rev*, 41, 401–425. Retrieved from https://doi.org/10.1080/05704920600726175

Dauvergne, P. (2018). Why is the global governance of plastic failing the oceans? *Global Environmental Change*, 51, 22–31. Retrieved from https://doi.org/https://doi.org/10.1016/j.gloenvcha.2018.05.002

Dawes, E. A. (1988). Polyhydroxybutyrate: an intriguing biopolymer. *Bioscience Reports*, 8(6), 537–547. Retrieved from https://doi.org/10.1007/bf01117332

de Koning, G. J. M. M., Kellerhals, M., Van Meurs, C., & Witholt, B. (1997). A process for the recovery of poly(hydroxyalkanoates) from Pseudomonads Part 2: Process development and economic evaluation. *Bioprocess Engineering*, 17(1), 15–21. Retrieved from https://doi.org/10.1007/s004490050346

Demuner, R. B., Soares Santos Maia, J. G., Secchi, A. R., Melo, P. A., do Carmo, R. W., & Gusmão, G. S. (2019). Modeling of Catalyst Deactivation in Bioethanol Dehydration Reactor. *Industrial & Engineering Chemistry Research*, 58(8), 2717–2726. Retrieved from https://doi.org/10.1021/acs.iecr.8b05699

Dey, S., & Tribedi, P. (2018). Microbial functional diversity plays an important role in the degradation of polyhydroxybutyrate (PHB) in soil. *3 Biotech*, 8(3), 171. Retrieved from https://doi.org/10.1007/s13205-018-1201-7

Dong, W., Zou, B., Yan, Y., Ma, P., & Chen, M. (2013). Effect of Chain-Extenders on the Properties and Hydrolytic Degradation Behavior of the Poly(lactide)/ Poly(butylene adipate-co-terephthalate) Blends. *International Journal of Molecular Sciences*, 14(10), 20189–20203. Retrieved from https://doi.org/10.3390/ijms141020189

Dorado, M. P., Lin, S. K. C., Koutinas, A., Du, C., Wang, R., & Webb, C. (2009). Cereal-based biorefinery development: Utilisation of wheat milling by-products for the production of succinic acid. *Journal of Biotechnology*, 143(1), 51–59. Retrieved from https://doi.org/https://doi.org/10.1016/j.jbiotec.2009.06.009

EC. (2006). Thematic strategy for soil protection. In: *Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee and the Committee of the Regions. Plastics - the Facts 2021*. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A52006DC0231

El-Abd, M. A. E.-H., El-Sheikh, H. H., Desouky, S., & Shehab, A. (2017). Identification, Biodegradation and bio-evaluation of biopolymer produced from Bacillus thuringenesis. *Journal of Applied Pharmaceutical Science*, 7, 103–110. Retrieved from https://doi.org/10.7324/JAPS.2017.70414

EN 17033. (2018). Plastics - Biodegradable mulch films for use in agriculture and horticulture -

Requirements and test methods. *European Standard*

Enescu, D., Frache, A., & Geobaldo, F. (2015). Formation and oxygen diffusion barrier properties of fish gelatin/natural sodium montmorillonite clay self-assembled multilayers onto the biopolyester surface. *Royal Society of Chemistry Advances*, 5, 61465–61480. Retrieved from https://doi.org/10.1039/C5RA11283D

Erkske, D., Viskere, I., Dzene, A., Tupureina, V., & Savenkova, L. (2006). Biobased polymer composites for films and coatings. *Proceedings of the Estonian Academy of Sciences*, 55, 70–77.

Fayyazbakhsh, A., Koutný, M., Kalendová, A., Šašinková, D., Julinová, M., & Kadlečková, M. (2022). Selected Simple Natural Antimicrobial Terpenoids as Additives to Control Biodegradation of Polyhydroxy Butyrate. *International Journal of Molecular Sciences*, 23(22). Retrieved from https://doi.org/10.3390/ijms232214079

Fernandes, M., Salvador, A., Alves, M. M., & Vicente, A. A. (2020). Factors affecting polyhydroxyalkanoates biodegradation in soil. *Polymer Degradation and Stability*, 182, 109408. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2020.109408

Forte, A., Zucaro, A., Basosi, R., & Fierro, A. (2016). LCA of 1,4-Butanediol Produced via Direct Fermentation of Sugars from Wheat Straw Feedstock within a Territorial Biorefinery. *Materials*, 9(7). Retrieved from https://doi.org/10.3390/ma9070563

Freitas, A. L. P. de L., Tonini Filho, L. R., Calvão, P. S., & Souza, A. M. C. de. (2017). Effect of montmorillonite and chain extender on rheological, morphological and biodegradation behavior of PLA/PBAT blends. *Polymer Testing*, 62, 189–195. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2017.06.030

Frey, S. D., Elliott, E. T., Paustian, K., & Peterson, G. A. (2000). Fungal translocation as a mechanism for soil nitrogen inputs to surface residue decomposition in a no-tillage agroecosystem. *Soil Biology and Biochemistry*, 32(5), 689–698. Retrieved from https://doi.org/https://doi.org/10.1016/S0038-0717(99)00205-9

Fu, Y., Wu, G., Bian, X., Zeng, J., & Weng, Y. (2020). Biodegradation Behavior of Poly(Butylene Adipate-Co-Terephthalate) (PBAT), Poly(Lactic Acid) (PLA), and Their Blend in Freshwater with Sediment. *Molecules*, 25(17). Retrieved from https://doi.org/10.3390/molecules25173946

Gallardo-Cervantes, M., González-García, Y., Pérez-Fonseca, A. A., González-López, M. E., Manríquez-González, R., Rodrigue, D., & Robledo-Ortíz, J. R. (2021). Biodegradability and improved mechanical performance of polyhydroxyalkanoates/agave fiber biocomposites compatibilized by different strategies. *Journal of Applied Polymer Science*, 138(15), 50182. Retrieved from https://doi.org/https://doi.org/10.1002/app.50182

Gautier, E., Fuertes, P., Cassagnau, P., Pascault, J.-P., & Fleury, E. (2009). Synthesis and rheology of biodegradable poly(glycolic acid) prepared by melt ring-opening polymerization of glycolide. *Journal of Polymer Science Part A: Polymer Chemistry*, 47(5), 1440–1449. Retrieved from https://doi.org/https://doi.org/10.1002/pola.23253

Gazvoda, L., Višić, B., Spreitzer, M., & Vukomanović, M. (2021). Hydrophilicity Affecting the Enzyme-Driven Degradation of Piezoelectric Poly-l-Lactide Films. *Polymers*, 13(11). Retrieved from https://doi.org/10.3390/polym13111719

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, 3(7). Retrieved 24 January 2022 from https://doi.org/10.1126/sciadv.1700782

Göktürk, E., Pemba, A. G., & Miller, S. A. (2015). Polyglycolic acid from the direct polymerization of renewable C1 feedstocks. *Polymer Chemistry*, 6(21), 3918–3925. Retrieved from https://doi.org/10.1039/C5PY00230C

Gomaa, Y. A., El-Khordagui, L. K., Boraei, N. A., & Darwish, I. A. (2010). Chitosan microparticles incorporating a hydrophilic sunscreen agent. *Carbohydrate Polymers*, 81(2), 234–242. Retrieved from

https://doi.org/https://doi.org/10.1016/j.carbpol.2010.02.024

Gomes, A., & Korf, B. (2018). Genetic Testing Techniques. In *Pediatric Cancer Genetics* (pp. 47–64). Retrieved from https://doi.org/10.1016/B978-0-323-48555-5.00005-3

Gómez, E. F., & Michel, F. C. (2013). Biodegradability of conventional and bio-based plastics and natural fiber composites during composting, anaerobic digestion and long-term soil incubation. *Polymer Degradation and Stability*, 98(12), 2583–2591. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2013.09.018

Gonçalves, S P C, Martins-Franchetti, S. M., & Chinaglia, D. L. (2009). Biodegradation of the Films of PP, PHBV and Its Blend in Soil. *Journal of Polymers and the Environment*, 17(4), 280. Retrieved from https://doi.org/10.1007/s10924-009-0150-y

Gonçalves, Suely Patrícia Costa, Strauss, M., & Martinez, D. S. T. (2018). The Positive Fate of Biochar Addition to Soil in the Degradation of PHBV-Silver Nanoparticle Composites. *Environmental Science & Technology*, 52(23), 13845–13853. Retrieved from https://doi.org/10.1021/acs.est.8b01524

Gu, J.-D. (2003). Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. *International Biodeterioration & Biodegradation*, 52(2), 69–91. Retrieved from https://doi.org/https://doi.org/10.1016/S0964-8305(02)00177-4

Gualandi, C., Soccio, M., Saino, E., Focarete, M. L., Lotti, N., Munari, A., … Visai, L. (2012). Easily synthesized novel biodegradable copolyesters with adjustable properties for biomedical applications. *Soft Matter*, 8(20), 5466–5476. Retrieved from https://doi.org/10.1039/C2SM25308A

Gundewadi, G., Rudra, S. G., Sarkar, D. J., & Singh, D. (2018). Nanoemulsion based alginate organic coating for shelf life extension of okra. *Food Packaging and Shelf Life*, 18, 1–12. Retrieved from https://doi.org/https://doi.org/10.1016/j.fpsl.2018.08.002

Günter, E. A., Popeyko, O. V, Belozerov, V. S., Martinson, E. A., & Litvinets, S. G. (2020). Physicochemical and swelling properties of composite gel microparticles based on alginate and callus cultures pectins with low and high degrees of methylesterification. *International Journal of Biological Macromolecules*, 164, 863–870. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2020.07.189

Ha, C.-S., & Cho, W.-J. (2002). Miscibility, properties, and biodegradability of microbial polyester containing blends. *Progress in Polymer Science*, 27(4), 759–809. Retrieved from https://doi.org/https://doi.org/10.1016/S0079-6700(01)00050-8

Haider, T. P., Völker, C., Kramm, J., Landfester, K., & Wurm, F. R. (2019). Plastics of the Future? The Impact of Biodegradable Polymers on the Environment and on Society. *Angewandte Chemie International Edition*, 58(1), 50–62. Retrieved from https://doi.org/https://doi.org/10.1002/anie.201805766

Han, J., Hou, J., Liu, H., Cai, S., Feng, B., Zhou, J., & Xiang, H. (2010). Wide Distribution among Halophilic Archaea of a Novel Polyhydroxyalkanoate Synthase Subtype with Homology to Bacterial Type III Synthases. *Applied and Environmental Microbiology*, 76, 7811–7819. Retrieved from https://doi.org/10.1128/AEM.01117-10

Han, Y., Teng, Y., Wang, X., Ren, W., Wang, X., Luo, Y., … Christie, P. (2021). Soil Type Driven Change in Microbial Community Affects Poly(butylene adipate- co-terephthalate) Degradation Potential. *Environmental Science and Technology*, 55(8), 4648–4657. Retrieved from https://doi.org/10.1021/acs.est.0c04850

Hiraishi, T., Komiya, N., Matsumoto, N., Abe, H., Fujita, M., & Maeda, M. (2010). Degradation and Adsorption Characteristics of PHB Depolymerase As Revealed by Kinetics of Mutant Enzymes with Amino Acid Substitution in Substrate-Binding Domain. *Biomacromolecules*, 11(1), 113–119. Retrieved from https://doi.org/10.1021/bm900967a

Hirsch, P., Gallikowski, C. A., Siebert, J., Peissl, K., Kroppenstedt, R., Schumann, P., … Anderson, R. (2004). Deinococcus frigens sp. nov., Deinococcus saxicola sp. nov., and Deinococcus marmoris sp.

nov., low temperature and draught-tolerating, UV-resistant bacteria from continental Antarctica. *Systematic and Applied Microbiology*, 27(6), 636–645. Retrieved from https://doi.org/10.1078/0723202042370008

Hong, K., Sun, S., Tian, W., Chen, G. Q., & Huang, W. (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology*, 51(4), 523–526. Retrieved from https://doi.org/10.1007/s002530051427

Hu, Xianwen, Du, Y., Tang, Y., Wang, Q., Feng, T., Yang, J., & Kennedy, J. F. (2007). Solubility and property of chitin in NaOH/urea aqueous solution. *Carbohydrate Polymers*, 70(4), 451–458. Retrieved from https://doi.org/https://doi.org/10.1016/j.carbpol.2007.05.002

Hu, Xiaoping, Thumarat, U., Zhang, X., Tang, M., & Kawai, F. (2010). Diversity of polyester-degrading bacteria in compost and molecular analysis of a thermoactive esterase from Thermobifida alba AHK119. *Applied Microbiology and Biotechnology*, 87(2), 771–779. Retrieved from https://doi.org/10.1007/s00253-010-2555-x

Huang, X., Li, C., Zheng, L., Zhang, D., Guan, G., & Xiao, Y. (2009). Synthesis, characterization and properties of biodegradable poly(butylene succinate)-block-poly(propylene glycol) segmented copolyesters. *Polymer International*, 58(8), 893–899. Retrieved from https://doi.org/https://doi.org/10.1002/pi.2609

Husárová, L., Pekařová, S., Stloukal, P., Kucharzcyk, P., Verney, V., Commereuc, S., … Koutny, M. (2014). Identification of important abiotic and biotic factors in the biodegradation of poly(l-lactic acid). *International Journal of Biological Macromolecules*, 71, 155–162. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2014.04.050

Iggui, K., Le Moigne, N., Kaci, M., Cambe, S., Degorce-Dumas, J. R., & Bergeret, A. (2015). A biodegradation study of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/organoclay nanocomposites in various environmental conditions. *Polymer Degradation and Stability*, 119, 77–86. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.05.002

ISO 17556. (2019). Plastics e Determination of the Ultimate Aerobic Biodegradability of Plastic Materials in Soil by Measuring the Oxygen Demand in a Respirometer or the Amount of Carbon Dioxide Evolved. *International Organization for Standardization*

ISO 472. (2013). Plastics — Vocabulary. *International Organization for Standardization*

Itävaara, M., & Vikman, M. (1995). A simple screening test for studying the biodegradability of insoluble polymers. *Chemosphere*, 31(11), 4359–4373. Retrieved from https://doi.org/https://doi.org/10.1016/0045-6535(95)00304-Q

Jain, R., & Tiwari, A. (2015). Biosynthesis of planet friendly bioplastics using renewable carbon source. *Journal of Environmental Health Science & Engineering*, 13, 1–11. Retrieved from https://doi.org/10.1186/s40201-015-0165-3

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., … Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, 347(6223), 768 LP – 771. Retrieved from https://doi.org/10.1126/science.1260352

Jendrossek, D., Schirmer, A., & Schlegel, H. G. (1996). Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology*, 46(5), 451–463. Retrieved from https://doi.org/10.1007/s002530050844

Jeszeova, L., Puskarova, A., Buckova, M., Krakova, L., Grivalsky, T., Danko, M., … Pangallo, D. (2018). Microbial communities responsible for the degradation of poly(lactic acid)/poly(3-hydroxybutyrate) blend mulches in soil burial respirometric tests. *World Journal of Microbiology & Biotechnology*, 34(7), 101. Retrieved from https://doi.org/10.1007/s11274-018-2483-y

Jia, H., Zhang, M., Weng, Y., Zhao, Y., Li, C., & Kanwal, A. (2021). Degradation of poly(butylene adipate-co-terephthalate) by Stenotrophomonas sp. YCJ1 isolated from farmland soil. *Journal of Environmental*

Sciences (China), 103, 50–58. Retrieved from https://doi.org/10.1016/j.jes.2020.10.001

Jian, J., Xiangbin, Z., & Xianbo, H. (2020). An overview on synthesis, properties and applications of poly(butylene-adipate-co-terephthalate)–PBAT. *Advanced Industrial and Engineering Polymer Research*, 3(1), 19–26. Retrieved from https://doi.org/10.1016/j.aiepr.2020.01.001

Jiang, B., Chen, C., Liang, Z., He, S., Kuang, Y., Song, J., ... Hu, L. (2020). Lignin as a Wood-Inspired Binder Enabled Strong, Water Stable, and Biodegradable Paper for Plastic Replacement. *Advanced Functional Materials*, 30(4), 1906307. Retrieved from https://doi.org/https://doi.org/10.1002/adfm.201906307

Jiang, L., Wolcott, M. P., & Zhang, J. (2006a). Study of Biodegradable Polylactide/Poly(butylene adipate-co-terephthalate) Blends. *Biomacromolecules*, 7(1), 199–207. Retrieved from https://doi.org/10.1021/bm050581q

Jiang, S., Yang, Y., Ge, S., Zhang, Z., & Peng, W. (2018). Preparation and properties of novel flame-retardant PBS wood-plastic composites. *Arabian Journal of Chemistry*, 11(6), 844–857. Retrieved from https://doi.org/https://doi.org/10.1016/j.arabjc.2017.12.023

Jiang, X., Ramsay, J. A., & Ramsay, B. A. (2006b). Acetone extraction of mcl-PHA from Pseudomonas putida KT2440. *Journal of Microbiological Methods*, 67(2), 212–219. Retrieved from https://doi.org/https://doi.org/10.1016/j.mimet.2006.03.015

Johannes, R., C., B. P., & Erland, B. (2009). Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. *Applied and Environmental Microbiology*, 75(6), 1589–1596. Retrieved from https://doi.org/10.1128/AEM.02775-08

Joyyi, L., Ahmad Thirmizir, M. Z., Salim, M. S., Han, L., Murugan, P., Kasuya, K., ... Sudesh, K. (2017). Composite properties and biodegradation of biologically recovered P(3HB-co-3HHx) reinforced with short kenaf fibers. *Polymer Degradation and Stability*, 137, 100–108. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.004

Jung, I. L., Phyo, K. H., Kim, K. C., Park, H. K., & Kim, I. G. (2005). Spontaneous liberation of intracellular polyhydroxybutyrate granules in Escherichia coli. *Research in Microbiology*, 156(8), 865–873. Retrieved from https://doi.org/https://doi.org/10.1016/j.resmic.2005.04.004

Kale, G., Auras, R., Singh, S. P., & Narayan, R. (2007). Biodegradability of polylactide bottles in real and simulated composting conditions. *Polymer Testing*, 26(8), 1049–1061. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2007.07.006

Kapritchkoff, F. M., Viotti, A. P., Alli, R. C. P., Zuccolo, M., Pradella, J. G. C., Maiorano, A. E., ... Bonomi, A. (2006). Enzymatic recovery and purification of polyhydroxybutyrate produced by Ralstonia eutropha. *Journal of Biotechnology*, 122(4), 453–462. Retrieved from https://doi.org/10.1016/j.jbiotec.2005.09.009

Karamanlioglu, M., & Robson, G. D. (2013). The influence of biotic and abiotic factors on the rate of degradation of poly(lactic) acid (PLA) coupons buried in compost and soil. *Polymer Degradation and Stability*, 98(10), 2063–2071. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2013.07.004

Kasuya, K. I., Inoue, Y., & Doi, Y. (1996). Adsorption kinetics of bacterial PHB depolymerase on the surface of polyhydroxyalkanoate films. *International Journal of Biological Macromolecules*, 19(1), 35–40. Retrieved from https://doi.org/10.1016/0141-8130(96)01097-5

Kasuya, K. ichi, Ishii, N., Inoue, Y., Yazawa, K., Tagaya, T., Yotsumoto, T., ... Nagai, D. (2009). Characterization of a mesophilic aliphatic-aromatic copolyester-degrading fungus. *Polymer Degradation and Stability*, 94(8), 1190–1196. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.04.013

Katav, T., Liu, L., Traitel, T., Goldbart, R., Wolfson, M., & Kost, J. (2008). Modified pectin-based carrier for gene delivery: Cellular barriers in gene delivery course. *Journal of Controlled Release*, 130(2), 183–

191. Retrieved from https://doi.org/10.1016/j.jconrel.2008.06.002

Kawai, F., Oda, M., Tamashiro, T., Waku, T., Tanaka, N., Yamamoto, M., … Tanokura, M. (2014). A novel Ca^{2+}-activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from Saccharomonospora viridis AHK190. *Applied Microbiology and Biotechnology*, 98(24), 10053–10064. Retrieved from https://doi.org/10.1007/s00253-014-5860-y

Kicińska, A., Pomykała, R., & Izquierdo-Diaz, M. (2022). Changes in soil pH and mobility of heavy metals in contaminated soils. *European Journal of Soil Science*, 73(1), e13203. Retrieved from https://doi.org/https://doi.org/10.1111/ejss.13203

Kijchavengkul, T., Auras, R., Rubino, M., Alvarado, E., Camacho Montero, J. R., & Rosales, J. M. (2010a). Atmospheric and soil degradation of aliphatic-aromatic polyester films. *Polymer Degradation and Stability*, 95(2), 99–107. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.11.048

Kijchavengkul, T., Auras, R., Rubino, M., Ngouajio, M., & Fernandez, R. T. (2008a). Assessment of aliphatic-aromatic copolyester biodegradable mulch films. Part I: Field study. *Chemosphere*, 71(5), 942–953. Retrieved from https://doi.org/10.1016/j.chemosphere.2007.10.074

Kijchavengkul, T., Auras, R., Rubino, M., Ngouajio, M., & Fernandez, R. T. (2008b). Assessment of aliphatic-aromatic copolyester biodegradable mulch films. Part II: Laboratory simulated conditions. *Chemosphere*, 71(9), 1607–1616. Retrieved from https://doi.org/10.1016/j.chemosphere.2008.01.037

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2010b). Biodegradation and hydrolysis rate of aliphatic aromatic polyester. *Polymer Degradation and Stability*, 95(12), 2641–2647. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.07.018

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2011). Formulation selection of aliphatic aromatic biodegradable polyester film exposed to UV/solar radiation. *Polymer Degradation and Stability*, 96(10), 1919–1926. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2011.07.001

Kikuchi, Y., Oshita, Y., Mayumi, K., & Hirao, M. (2017). Greenhouse gas emissions and socioeconomic effects of biomass-derived products based on structural path and life cycle analyses: A case study of polyethylene and polypropylene in Japan. *Journal of Cleaner Production*, 167, 289–305. Retrieved from https://doi.org/https://doi.org/10.1016/j.jclepro.2017.08.179

Kim, D. Y., Yun, J., Kim, H., Bae, K. S., & Rhee, Y. (2002). Purification and Characterization of Poly(3-hydroxybutyrate) Depolymerase from a Fungal Isolate, Emericellopsis minima W2. *The Journal of Microbiology*, 40, 129–133. Retrieved from https://www.researchgate.net/publication/340829175_Purification_and_Characterization_of_Poly3-hydroxybutyrate_Depolymerase_from_a_Fungal_Isolate_Emericellopsis_minima_W2

Kim, J., Gupta, N. S., Bezek, L. B., Linn, J., Bejagam, K. K., Banerjee, S., … Lee, K.-S. (2023). Biodegradation Studies of Polyhydroxybutyrate and Polyhydroxybutyrate-co-Polyhydroxyvalerate Films in Soil. *International Journal of Molecular Sciences*, 24(8). Retrieved from https://doi.org/10.3390/ijms24087638

Kim, M.-N., Lee, A.-R., Yoon, J.-S., & Chin, I.-J. (2000). Biodegradation of poly(3-hydroxybutyrate), Sky-Green (R) and Mater-Bi (R) by fungi isolated from soils. *European Polymer Journal*, 36, 1677–1685. Retrieved from https://doi.org/10.1016/S0014-3057(99)00219-0

Kleeberg, I, Welzel, K., VandenHeuvel, J., Müller, R.-J., & Deckwer, W.-D. (2005). Characterization of a New Extracellular Hydrolase from Thermobifida fusca Degrading Aliphatic–Aromatic Copolyesters. *Biomacromolecules*, 6(1), 262–270. Retrieved from https://doi.org/10.1021/bm049582t

Kleeberg, Ilona, Hetz, C., Kroppenstedt, R. M., Müller, R. J., & Deckwer, W. D. (1998). Biodegradation of aliphatic-aromatic copolyesters by Thermomonospora fusca and other thermophilic compost isolates. *Applied and Environmental Microbiology*, 64(5), 1731–1735. Retrieved from

https://doi.org/10.1128/aem.64.5.1731-1735.1998

Knoll, M., Hamm, T. M., Wagner, F., Martinez, V., & Pleiss, J. (2009). The PHA Depolymerase Engineering Database: A systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinformatics*, 10, 89. Retrieved from https://doi.org/10.1186/1471-2105-10-89

Koenig, J. (2001). Infrared and Raman Spectroscopy of Polymers (Vol. 12(2), pp. 16–26). Retrieved from https://doi.org/10.1016/B978-044410031-3/50005-0

Koller, M., Atlic, A., De Sousa Dias, M., Reiterer, A., & Braunegg, G. (2009). Microbial PHA Production from Waste Raw Materials. In *Plastics from Bacteria: Natural Functions and Applications* (Vol. 14, pp. 85–119). Retrieved from https://doi.org/10.1007/978-3-642-03287-5_5

Kosorn, W., Sakulsumbat, M., Uppanan, P., Kaewkong, P., Chantaweroad, S., Jitsaard, J., … Janvikul, W. (2017). PCL/PHBV blended three dimensional scaffolds fabricated by fused deposition modeling and responses of chondrocytes to the scaffolds. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 105(5), 1141–1150. Retrieved from https://doi.org/10.1002/jbm.b.33658

Kozlov, P. V, & Burdygina, G. I. (1983). The structure and properties of solid gelatin and the principles of their modification. *Polymer*, 24(6), 651–666. Retrieved from https://doi.org/https://doi.org/10.1016/0032-3861(83)90001-0

Kramer, C., & Gleixner, G. (2008). Soil organic matter in soil depth profiles: Distinct carbon preferences of microbial groups during carbon transformation. *Soil Biology and Biochemistry*, 40(2), 425–433. Retrieved from https://doi.org/https://doi.org/10.1016/j.soilbio.2007.09.016

Kruyer, N. S., & Peralta-Yahya, P. (2017). Metabolic engineering strategies to bio-adipic acid production. *Current Opinion in Biotechnology*, 45, 136–143. Retrieved from https://doi.org/https://doi.org/10.1016/j.copbio.2017.03.006

Kulkarni, S. O., Kanekar, P. P., Jog, J. P., Patil, P. A., Nilegaonkar, S. S., Sarnaik, S. S., & Kshirsagar, P. R. (2011). Characterisation of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) produced by Halomonas campisalis (MCM B-1027), its biodegradability and potential application. *Bioresource Technology*, 102(11), 6625–6628. Retrieved from https://doi.org/https://doi.org/10.1016/j.biortech.2011.03.054

Kuntanoo, K., & Promkotra, S. (2013). Biodegradation of Polyhydroxybutyrate-Co- Hydroxyvalerate (PHBV) Blended with Natural Rubber in Soil Environment. *World Academy of Science, Engineering and Technology*, 7, 1799–1803. Retrieved from https://doi.org/10.5281/zenodo.1087696

Lammi, S., Gastaldi, E., Gaubiac, F., & Angellier-Coussy, H. (2019). How olive pomace can be valorized as fillers to tune the biodegradation of PHBV based composites. *Polymer Degradation and Stability*, 166, 325–333. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2019.06.010

Law, J. H., & Slepecky, R. A. (1961). Assay of poly-beta-hydroxybutyric acid. *Journal of Bacteriology*, 82(1), 33–36. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/13759651

Lee, S. Y., & Choi, J. (1999). Production and degradation of polyhydroxyalkanoates in waste environment. *Waste Management*, 19(2), 133–139. Retrieved from https://doi.org/https://doi.org/10.1016/S0956-053X(99)00005-7

Li, W.-J., Narancic, T., Kenny, S. T., Niehoff, P.-J., O'Connor, K., Blank, L. M., & Wierckx, N. (2020). Unraveling 1,4-Butanediol Metabolism in Pseudomonas putida KT2440. *Frontiers in Microbiology*, 11, 382. Retrieved from https://doi.org/10.3389/fmicb.2020.00382

Li, Z., Lin, H., Ishii, N., Chen, G.-Q., & Inoue, Y. (2007). Study of enzymatic degradation of microbial copolyesters consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates. *Polymer Degradation and Stability*, 92(9), 1708–1714. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2007.06.001

Lim, S.-P., Gan, S.-N., & Tan, I. K. P. (2005). Degradation of medium-chain-length polyhydroxyalkanoates in tropical forest and mangrove soils. *Applied Biochemistry and Biotechnology*, 126(1), 23–33. Retrieved from https://doi.org/10.1007/s12010-005-0003-7

Limpan, N., Prodpran, T., Benjakul, S., & Prasarpran, S. (2012). Influences of degree of hydrolysis and molecular weight of poly(vinyl alcohol) (PVA) on properties of fish myofibrillar protein/PVA blend films. *Food Hydrocolloids*, 29(1), 226–233. Retrieved from https://doi.org/https://doi.org/10.1016/j.foodhyd.2012.03.007

Limpisophon, K., Tanaka, M., & Osako, K. (2010). Characterisation of gelatin–fatty acid emulsion films based on blue shark (Prionace glauca) skin gelatin. *Food Chemistry*, 122(4), 1095–1101. Retrieved from https://doi.org/https://doi.org/10.1016/j.foodchem.2010.03.090

Liu, S., Moon, C. D., Zheng, N., Huws, S., Zhao, S., & Wang, J. (2022a). Opportunities and challenges of using metagenomic data to bring uncultured microbes into cultivation. *Microbiome*, 10(1), 76. Retrieved from https://doi.org/10.1186/s40168-022-01272-5

Liu, Wei, Du, H., Liu, H., Xie, H., Xu, T., Zhao, X., ... Si, C. (2020a). Highly Efficient and Sustainable Preparation of Carboxylic and Thermostable Cellulose Nanocrystals via FeCl3-Catalyzed Innocuous Citric Acid Hydrolysis. *ACS Sustainable Chemistry & Engineering*, 8(44), 16691–16700. Retrieved from https://doi.org/10.1021/acssuschemeng.0c06561

Liu, Wenying, Zhang, S., Yang, K., Yu, W., Shi, J., & Zheng, Q. (2022b). Preparation of graphene-modified PLA/PBAT composite monofilaments and its degradation behavior. *Journal of Materials Research and Technology*, 20, 3784–3795. Retrieved from https://doi.org/https://doi.org/10.1016/j.jmrt.2022.08.125

Liu, Wenyong, Liu, S., Wang, Z., Liu, J., Dai, B., Chen, Y., & Zeng, G. (2020b). Preparation and characterization of compatibilized composites of poly(butylene adipate-co-terephthalate) and thermoplastic starch by two-stage extrusion. *European Polymer Journal*, 122, 109369. Retrieved from https://doi.org/https://doi.org/10.1016/j.eurpolymj.2019.109369

Liu, Yalan, Zhan, Z., Ye, H., Lin, X., Yan, Y., & Zhang, Y. (2019). Accelerated biodegradation of PLA/PHB-blended nonwovens by a microbial community. *RSC Advances*, 9(18), 10386–10394. Retrieved from https://doi.org/10.1039/C8RA10591J

Liu, Yufei, Liu, S., Liu, Z., Lei, Y., Jiang, S., Zhang, K., ... Yu, J. (2021). Enhanced mechanical and biodegradable properties of PBAT/lignin composites via silane grafting and reactive extrusion. *Composites Part B: Engineering*, 220, 108980. Retrieved from https://doi.org/https://doi.org/10.1016/j.compositesb.2021.108980

Loos, K., Zhang, R., Pereira, I., Agostinho, B., Hu, H., Maniar, D., ... Sousa, A. F. (2020). A Perspective on PEF Synthesis, Properties, and End-Life. *Frontiers in Chemistry*, 8(585). Retrieved from https://www.frontiersin.org/articles/10.3389/fchem.2020.00585

López-Cuellar, M. R., Alba-Flores, J., Rodríguez, J. N. G., & Pérez-Guevara, F. (2011). Production of polyhydroxyalkanoates (PHAs) with canola oil as carbon source. *International Journal of Biological Macromolecules*, 48(1), 74–80. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2010.09.016

Lopez-Llorca, L. V, Colom Valiente, M. F., & Gascon, A. (1993). A study of biodegradation of poly-β-hydroxyalkanoate (PHA) films in soil using scanning electron microscopy. *Micron*, 24(1), 23–29. Retrieved from https://doi.org/https://doi.org/10.1016/0968-4328(93)90012-P

Lu, J., Tappel, R. C., & Nomura, C. T. (2009). Mini-review: Biosynthesis of poly(hydroxyalkanoates). *Polymer Reviews*, 49(3), 226–248. Retrieved from https://doi.org/10.1080/15583720903048243

Luo, S., & Netravali, A. N. (2003). A study of physical and mechanical properties of poly(hydroxybutyrate-co-hydroxyvalerate) during composting. *Polymer Degradation and Stability*, 80(1), 59–66. Retrieved from https://doi.org/https://doi.org/10.1016/S0141-3910(02)00383-X

Madbouly, S., Schrader, J., Srinivasan, G., Liu, K., McCabe, K., Grewell, D., ... Kessler, M. (2014). Biodegradation behavior of bacterial-based polyhydroxyalkanoate (PHA) and DDGS composites. *Green Chem.*, 16(4), 1911–1920. Retrieved from https://doi.org/10.1039/C3GC41503A

Madhumathi, K., Binulal, N. S., Nagahama, H., Tamura, H., Shalumon, K. T., Selvamurugan, N., ... Jayakumar, R. (2009). Preparation and characterization of novel beta-chitin-hydroxyapatite composite membranes for tissue engineering applications. *International Journal of Biological Macromolecules*, 44(1), 1–5. Retrieved from https://doi.org/10.1016/j.ijbiomac.2008.09.013

Madhumathi, K., Sudheesh Kumar, P. T., Abhilash, S., Sreeja, V., Tamura, H., Manzoor, K., ... Jayakumar, R. (2010). Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. *Journal of Materials Science: Materials in Medicine*, 21(2), 807–813. Retrieved from https://doi.org/10.1007/s10856-009-3877-z

Madison, L. L., & Huisman, G. W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiology and Molecular Biology Reviews*, 63(March), 21–53. Retrieved from https://doi.org/10.1128/mmbr.63.1.21-53.1999

Manna, A., & Paul, A. K. (2000). Degradation of microbial polyester poly(3-hydroxybutyrate) in environmental samples and in culture. *Biodegradation*, 11(5), 323–329. Retrieved from https://doi.org/10.1023/A:1011162624704

Marten, E., Müller, R. J., & Deckwer, W. D. (2005). Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic-aromatic copolyesters. *Polymer Degradation and Stability*, 88(3), 371–381. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2004.12.001

Martínez-Abad, A., Sánchez, G., Fuster, V., Lagaron, J. M., & Ocio, M. J. (2013). Antibacterial performance of solvent cast polycaprolactone (PCL) films containing essential oils. *Food Control*, 34(1), 214–220. Retrieved from https://doi.org/https://doi.org/10.1016/j.foodcont.2013.04.025

Masood, F., Yasin, T., & Hameed, A. (2014). Comparative oxo-biodegradation study of poly-3-hydroxybutyrate-co-3-hydroxyvalerate/polypropylene blend in controlled environments. *International Biodeterioration & Biodegradation*, 87, 1–8. Retrieved from https://doi.org/https://doi.org/10.1016/j.ibiod.2013.09.023

Matavulj, M., & Molitoris, H. P. (1992). Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. *FEMS Microbiology Reviews*, 9(2–4), 323–331. Retrieved from https://doi.org/10.1111/j.1574-6968.1992.tb05854.x

Mathew, A. P., Oksman, K., & Sain, M. (2005). Mechanical properties of biodegradable composites from poly lactic acid (PLA) and microcrystalline cellulose (MCC). *Journal of Applied Polymer Science*, 97(5), 2014–2025. Retrieved from https://doi.org/https://doi.org/10.1002/app.21779

Mercier, A., Gravouil, K., Aucher, W., Brosset-Vincent, S., Kadri, L., Colas, J., ... Ferreira, T. (2017). Fate of Eight Different Polymers under Uncontrolled Composting Conditions: Relationships Between Deterioration, Biofilm Formation, and the Material Surface Properties. *Environmental Science & Technology*, 51(4), 1988–1997. Retrieved from https://doi.org/10.1021/acs.est.6b03530

Mergaert, J., Anderson, C., Wouters, A., Swings, J., & Kersters, K. (1992). Biodegradation of polyhydroxyalkanoates. *FEMS Microbiology Letters*, 103(2), 317–321. Retrieved from https://doi.org/https://doi.org/10.1016/0378-1097(92)90325-I

Mergaert, J., & Swings, J. (1996). Biodiversity of microorganisms that degrade bacterial and synthetic polyesters. *Journal of Industrial Microbiology*, 17(5), 463–469. Retrieved from https://doi.org/10.1007/BF01574777

Mergaert, J., Webb, A., Anderson, C., Wouters, A., & Swings, J. (1993). Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. *Applied and Environmental Microbiology*, 59(10), 3233–3238. Retrieved from https://doi.org/10.1128/aem.59.10.3233-3238.1993

Mesquita, P., Araújo, R., Andrade, D., Carvalho, L., Alves, T., & Barbosa, R. (2016). Evaluation of Biodegradation of PHB/PP-G-MA/Vermiculite Bionanocomposites. *Materials Science Forum*, 869, 298–302. Retrieved from https://doi.org/10.4028/www.scientific.net/MSF.869.298

Meyer-Cifuentes, I. E., Werner, J., Jehmlich, N., Will, S. E., Neumann-Schaal, M., & Öztürk, B. (2020). Synergistic biodegradation of aromatic-aliphatic copolyester plastic by a marine microbial consortium. *Nature Communications*, 11(1). Retrieved from https://doi.org/10.1038/s41467-020-19583-2

Mitsumata, T., Suemitsu, Y., Fujii, K., Fujii, T., Taniguchi, T., & Koyama, K. (2003). pH-response of chitosan, κ-carrageenan, carboxymethyl cellulose sodium salt complex hydrogels. *Polymer*, 44(23), 7103–7111. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymer.2003.09.001

Mofokeng, J. P., & Luyt, A. S. (2015). Morphology and thermal degradation studies of melt-mixed poly(lactic acid) (PLA)/poly(ε-caprolactone) (PCL) biodegradable polymer blend nanocomposites with TiO2 as filler. *Polymer Testing*, 45, 93–100. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2015.05.007

Montagna, L. (2016). Evaluating the biodegradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/carbon nanotube nanocomposites in soil. *Conference: BCCM 3 - Brazilian Conference on Composites Materials*. Retrieved from https://www.researchgate.net/publication/319785378_evaluating_the_biodegradation_of_poly3-hydroxybutyrate-co-3 hydroxyvaleratecarbon_nanotube_nanocomposites_in_soil

Morais, C., Freitas, F., Cruz, M. V, Paiva, A., Dionísio, M., & Reis, M. A. M. (2014). Conversion of fat-containing waste from the margarine manufacturing process into bacterial polyhydroxyalkanoates. *International Journal of Biological Macromolecules*, 71, 68–73. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2014.04.044

Mousavioun, P., George, G. A., & Doherty, W. O. S. (2012). Environmental degradation of lignin/poly(hydroxybutyrate) blends. *Polymer Degradation and Stability*, 97(7), 1114–1122. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.04.004

Moustafa, H., Guizani, C., Dupont, C., Martin, V., Jeguirim, M., & Dufresne, A. (2017). Utilization of Torrefied Coffee Grounds as Reinforcing Agent To Produce High-Quality Biodegradable PBAT Composites for Food Packaging Applications. *ACS Sustainable Chemistry & Engineering*, 5(2), 1906–1916. Retrieved from https://doi.org/10.1021/acssuschemeng.6b02633

Mukhiddinov, Z. K., Khalikov, D. K., Abdusamiev, F. T., & Avloev, C. C. (2000). Isolation and structural characterization of a pectin homo and ramnogalacturonan. *Talanta*, 53(1), 171–176. Retrieved from https://doi.org/https://doi.org/10.1016/S0039-9140(00)00456-2

Mulbry, W., Reeves, J. B., & Millner, P. (2012). Use of mid- and near-infrared spectroscopy to track degradation of bio-based eating utensils during composting. *Bioresource Technology*, 109, 93–97. Retrieved from https://doi.org/https://doi.org/10.1016/j.biortech.2012.01.029

Müller, C. A., Perz, V., Provasnek, C., Quartinello, F., Guebitz, G. M., & Berg, G. (2017). Discovery of polyesterases from mossassociated microorganisms. *Applied and Environmental Microbiology*, 83(4). Retrieved from https://doi.org/10.1128/AEM.02641-16

Müller, R.-J. (2005). Biodegradability of Polymers: Regulations and Methods for Testing. *Biopolymers Online*, 365–374. Retrieved from https://doi.org/doi:10.1002/3527600035.bpola012

Müller, R.-J., Schrader, H., Profe, J., Dresler, K., & Deckwer, W.-D. (2005). Enzymatic Degradation of Poly(ethylene terephthalate): Rapid Hydrolyse using a Hydrolase from T. fusca. *Macromolecular Rapid Communications*, 26(17), 1400–1405. Retrieved from https://doi.org/https://doi.org/10.1002/marc.200500410

Munarin, F., Guerreiro, S. G., Grellier, M. A., Tanzi, M. C., Barbosa, M. A., Petrini, P., & Granja, P. L. (2011). Pectin-based injectable biomaterials for bone tissue engineering. *Biomacromolecules*, 12(3), 568–577. Retrieved from https://doi.org/10.1021/bm101110x

Muniyasamy, S., Ofosu, O., John, M. J., & Anandjiwala, R. D. (2016). Mineralization of Poly(lactic acid) (PLA), Poly(3-hydroxybutyrate-co-valerate) (PHBV) and PLA/PHBV Blend in Compost and Soil Environments. *Journal of Renewable Materials*, 4(2), 133–145. Retrieved from https://doi.org/10.7569/JRM.2016.634104

Muroi, F., Tachibana, Y., Soulenthone, P., Yamamoto, K., Mizuno, T., Sakurai, T., ... Kasuya, K. (2017). Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the aerobic mesophilic bacterium Bacillus pumilus. *Polymer Degradation and Stability*, 137, 11–22. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.006

Mutalik, S., Manoj, K., Reddy, M. S., Kushtagi, P., Usha, A. N., Anju, P., ... Udupa, N. (2008). Chitosan and enteric polymer based once daily sustained release tablets of aceclofenac: in vitro and in vivo studies. *AAPS PharmSciTech*, 9(2), 651–659. Retrieved from https://doi.org/10.1208/s12249-008-9075-3

Muthuraj, R., Misra, M., & Mohanty, A. K. (2015). Hydrolytic degradation of biodegradable polyesters under simulated environmental conditions. *Journal of Applied Polymer Science*, 132(27). Retrieved from https://doi.org/10.1002/app.42189

Najmadeen, H., Mohammad, O., & Mohamed-Amin, H. (2010). Effects of Soil Texture on Chemical Compositions, Microbial Populations and Carbon Mineralization in Soil. *J. Exp. Biol*, 6(1), 59–64. Retrieved from http://www.egyseb.org

Nakajima-Kambe, T., Ichihashi, F., Matsuzoe, R., Kato, S., & Shintani, N. (2009a). Degradation of aliphatic-aromatic copolyesters by bacteria that can degrade aliphatic polyesters. *Polymer Degradation and Stability*, 94(11), 1901–1905. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.08.006

Nakajima-Kambe, T., Toyoshima, K., Saito, C., Takaguchi, H., Akutsu-Shigeno, Y., Sato, M., ... Uchiyama, H. (2009b). Rapid monomerization of poly(butylene succinate)-co-(butylene adipate) by Leptothrix sp. *Journal of Bioscience and Bioengineering*, 108(6), 513–516. Retrieved from https://doi.org/https://doi.org/10.1016/j.jbiosc.2009.05.018

Nannipieri, P., Ascher-Jenull, J., Ceccherini, M. T., Landi, L., Pietramellara, G., & Renella, G. (2017). Microbial diversity and soil functions. *European Journal of Soil Science*, 68, 12–26. Retrieved from https://doi.org/10.1111/ejss.4_12398

Navarre, N., Mogollón, J. M., Tukker, A., & Barbarossa, V. (2022). Recycled plastic packaging from the Dutch food sector pollutes Asian oceans. *Resources, Conservation and Recycling*, 185, 106508. Retrieved from https://doi.org/https://doi.org/10.1016/j.resconrec.2022.106508

Nawaz, A., Hasan, F., & Shah, A. A. (2015). Degradation of poly(ε-caprolactone) (PCL) by a newly isolated Brevundimonas sp. strain MRL-AN1 from soil. *FEMS Microbiology Letters*, 362(1), 1–7. Retrieved from https://doi.org/10.1093/femsle/fnu004

NF U52-001. (2005). Biodegradable Materials for Use in Agriculture and Horticulture mulching Products-Requirements and Test Methods. *Association Francaise de Normalisation*

Nguyenhuynh, T., Yoon, L. W., Chow, Y. H., & Chua, A. S. M. (2021). An insight into enrichment strategies for mixed culture in polyhydroxyalkanoate production: feedstocks, operating conditions and inherent challenges. *Chemical Engineering Journal*, 420(P3), 130488. Retrieved from https://doi.org/10.1016/j.cej.2021.130488

Nikolić, M. A. L., Gauthier, E., Colwell, J. M., Halley, P., Bottle, S. E., Laycock, B., & Truss, R. (2017). The challenges in lifetime prediction of oxodegradable polyolefin and biodegradable polymer films. *Polymer Degradation and Stability*, 145, 102–119. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2017.07.018

Nishida, H., & Tokiwa, Y. (1993a). Distribution of poly(β-hydroxybutyrate) and poly(ε-caprolactone)aerobic degrading microorganisms in different environments. *Journal of Environmental Polymer Degradation*,

1(3), 227–233. Retrieved from https://doi.org/10.1007/BF01458031

Nishida, H., & Tokiwa, Y. (1993b). Effects of higher-order structure of poly(3-hydroxybutyrate) on its biodegradation. II. Effects of crystal structure on microbial degradation. *Journal of Environmental Polymer Degradation*, 1(1), 65–80. Retrieved from https://doi.org/10.1007/BF01457654

Nishide, H., Toyota, K., & Kimura, M. (1999). Effects of soil temperature and anaerobiosis on degradation of biodegradable plastics in soil and their degrading microorganisms. *Soil Science and Plant Nutrition*, 45(4), 963–972. Retrieved from https://doi.org/10.1080/00380768.1999.10414346

Nishioka, M., Nakai, K., Miyake, M., Asada, Y., & Taya, M. (2001). Production of poly-β-hydroxybutyrate by thermophilic cyanobacterium, Synechococcus sp. MA19, under phosphate-limited conditions. *Biotechnology Letters*, 23(14), 1095–1099. Retrieved from https://doi.org/10.1023/A:1010551614648

Nobes, G. A. R., Marchessault, R. H., Chanzy, H., Briese, B. H., & Jendrossek, D. (1996). Splintering of Poly(3-hydroxybutyrate) Single Crystals by PHB-Depolymerase A from Pseudomonas lemoignei. *Macromolecules*, 29(26), 8330–8333. Retrieved from https://doi.org/10.1021/ma961219u

Nur Hanani, Z. A., Roos, Y. H., & Kerry, J. P. (2014). Use and application of gelatin as potential biodegradable packaging materials for food products. *International Journal of Biological Macromolecules*, 71, 94–102. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2014.04.027

Oda, Y., Asari, H., Urakami, T., & Tonomura, K. (1995). Microbial degradation of poly(3-hydroxybutyrate) and polycaprolactone by filamentous fungi. *Journal of Fermentation and Bioengineering*, 80(3), 265–269. Retrieved from https://doi.org/10.1016/0922-338X(95)90827-M

Oechslein, C. R., Fricker, G., & Kissel, T. (1996). Nasal delivery of octreotide: Absorption enhancement by particulate carrier systems. *International Journal of Pharmaceutics*, 139(1), 25–32. Retrieved from https://doi.org/https://doi.org/10.1016/0378-5173(96)04569-3

Ohkita, T., & Lee, S.-H. (2006). Thermal degradation and biodegradability of poly (lactic acid)/corn starch biocomposites. *Journal of Applied Polymer Science*, 100(4), 3009–3017. Retrieved from https://doi.org/10.1002/app.23425

Ol'khov, A. A., Iordanskii, A. L., & Danko, T. P. (2015). Morphology of poly(3-hydroxybutyrate)–polyvinyl alcohol extrusion films, 35(8), 765–771. Retrieved from https://doi.org/doi:10.1515/polyeng-2014-0202

Ong, S. Y., & Sudesh, K. (2016). Effects of polyhydroxyalkanoate degradation on soil microbial community. *Polymer Degradation and Stability*, 131, 9–19. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2016.06.024

Pagga, U. (1998). Biodegradability and compostability of polymeric materials in the context of the European packaging regulation. *Polymer Degradation and Stability*, 59(1), 371–376. Retrieved from https://doi.org/https://doi.org/10.1016/S0141-3910(97)00192-4

Palsikowski, P. A., Kuchnier, C. N., Pinheiro, I. F., & Morales, A. R. (2018). Biodegradation in Soil of PLA/PBAT Blends Compatibilized with Chain Extender. *Journal of Polymers and the Environment*, 26(1), 330–341. Retrieved from https://doi.org/10.1007/s10924-017-0951-3

Pederson, E. N., McChalicher, C. W. J., & Srienc, F. (2006). Bacterial Synthesis of PHA Block Copolymers. *Biomacromolecules*, 7(6), 1904–1911. Retrieved from https://doi.org/10.1021/bm0510101

Pérez-Arauz, A. O., Aguilar-Rabiela, A. E., Vargas-Torres, A., Rodríguez-Hernández, A.-I., Chavarría-Hernández, N., Vergara-Porras, B., & López-Cuellar, M. R. (2019). Production and characterization of biodegradable films of a novel polyhydroxyalkanoate (PHA) synthesized from peanut oil. *Food Packaging and Shelf Life*, 20, 100297. Retrieved from https://doi.org/https://doi.org/10.1016/j.fpsl.2019.01.001

Pervaiz, M., Faruq, M., Jawaid, M., & Sain, M. (2016). Polyamides: Developments and Applications

Towards Next-Generation Engineered Plastics. *Current Organic Synthesis*, 13, 1. Retrieved from https://doi.org/10.2174/1570179413666160831112159

Perz, V., Baumschlager, A., Bleymaier, K., Zitzenbacher, S., Hromic, A., Steinkellner, G., ... Guebitz, G. M. (2016b). Hydrolysis of synthetic polyesters by Clostridium botulinum esterases. *Biotechnology and Bioengineering*, 113(5), 1024–1034. Retrieved from https://doi.org/https://doi.org/10.1002/bit.25874

Perz, V., Bleymaier, K., Sinkel, C., Kueper, U., Bonnekessel, M., Ribitsch, D., & Guebitz, G. M. (2016a). Substrate specificities of cutinases on aliphatic–aromatic polyesters and on their model substrates. *New Biotechnology*, 33(2), 295–304. Retrieved from https://doi.org/https://doi.org/10.1016/j.nbt.2015.11.004

Perz, V., Hromic, A., Baumschlager, A., Steinkellner, G., Pavkov-Keller, T., Gruber, K., ... Guebitz, G. M. (2016c). An Esterase from Anaerobic Clostridium hathewayi Can Hydrolyze Aliphatic–Aromatic Polyesters. *Environmental Science & Technology*, 50(6), 2899–2907. Retrieved from https://doi.org/10.1021/acs.est.5b04346

Phadke, S., Salvador, A. F., Alves, J. I., Bretschger, O., Alves, M. M., & Pereira, M. A. (2017). Harnessing the Power of PCR Molecular Fingerprinting Methods and Next Generation Sequencing for Understanding Structure and Function in Microbial Communities.0. *Methods in Molecular Biology (Clifton, N.J.)*, 1620, 225–248. Retrieved from https://doi.org/10.1007/978-1-4939-7060-5_16

Pietrini, M., Roes, L., Patel, M. K., & Chiellini, E. (2007). Comparative life cycle studies on poly(3-hydroxybutyrate)-based composites as potential replacement for conventional petrochemical plastics. *Biomacromolecules*, 8(7), 2210–2218. Retrieved from https://doi.org/10.1021/bm0700892

Plackett, D., & Katiyar, V. (2011). Biopolymers - New Materials for Sustainable Films and Coatings (pp. 301–315). Retrieved from https://doi.org/10.1002/9781119994312.ch14

Plastics, E. (2021). An Analysis of European Plastics Production, Demand and Waste Data, Plastics Europe-Association of Plastics Manufacturers. *Plastics - the Facts 2021*. Retrieved from http://www.plasticseurope.org/

Poli, A., Di Donato, P., Abbamondi, G. R., & Nicolaus, B. (2011). Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by Archaea. *Archaea*, 2011, 1–13. Retrieved from https://doi.org/10.1155/2011/693253

Pouteau, C., Dole, P., Cathala, B., Averous, L., & Boquillon, N. (2003). Antioxidant properties of lignin in polypropylene. *Polymer Degradation and Stability*, 81(1), 9–18. Retrieved from https://doi.org/https://doi.org/10.1016/S0141-3910(03)00057-0

Prudnikova, S. V., Vinogradova, O. N., & Trusova, M. Y. (2017). Specific character of bacterial biodegradation of polyhydroxyalkanoates with different chemical structure in soil. *Doklady Biochemistry and Biophysics*, 473, 94–97. Retrieved from https://doi.org/10.1134/S1607672917010185

Puscaselu, R., Gutt, G., & Amariei, S. (2019). Rethinking the Future of Food Packaging: Biobased Edible Films for Powdered Food and Drinks. *Molecules (Basel, Switzerland)*, 24(17). Retrieved from https://doi.org/10.3390/molecules24173136

Qi, R., Jones, D. L., Liu, Q., Liu, Q., Li, Z., & Yan, C. (2021). Field test on the biodegradation of poly(butylene adipate-co-terephthalate) based mulch films in soil. *Polymer Testing*, 93, 107009. Retrieved from https://doi.org/10.1016/j.polymertesting.2020.107009

Qiu, Z., Ikehara, T., & Nishi, T. (2003). Poly(hydroxybutyrate)/poly(butylene succinate) blends: miscibility and nonisothermal crystallization. *Polymer*, 44(8), 2503–2508. Retrieved from https://doi.org/https://doi.org/10.1016/S0032-3861(03)00150-2

Qu, R., Liu, G., Yue, M., Wang, G., Peng, C., Wang, K., & Gao, X. (2023). Soil temperature, microbial biomass and enzyme activity are the critical factors affecting soil respiration in different soil layers in

Ziwuling Mountains, China. *Frontiers in Microbiology*, 14, 1–12. Retrieved from https://www.frontiersin.org/articles/10.3389/fmicb.2023.1105723

Rahman, M. Z., Rahman, M., Mahbub, T., Ashiquzzaman, M., Sagadevan, S., & Hoque, M. E. (2023). Advanced biopolymers for automobile and aviation engineering applications. *Journal of Polymer Research*, 30(3), 106. Retrieved from https://doi.org/10.1007/s10965-023-03440-z

Rainey, F. A., Ray, K., Ferreira, M., Gatz, B. Z., Nobre, M. F., Bagaley, D., ... da Costa, M. S. (2005). Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran Desert soil and description of nine new species of the genus Deinococcus obtained from a single soil sample. *Applied and Environmental Microbiology*, 71(9), 5225–5235. Retrieved from https://doi.org/10.1128/AEM.71.9.5225-5235.2005

Ramos, T. B., Horta, A., Gonçalves, M. C., Pires, F. P., Duffy, D., & Martins, J. C. (2017). The INFOSOLO database as a first step towards the development of a soil information system in Portugal. *CATENA*, 158, 390–412. Retrieved from https://doi.org/https://doi.org/10.1016/j.catena.2017.07.020

Ramsay, J. A., Berger, E., Voyer, R., Chavarie, C., & Ramsay, B. A. (1994). Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Techniques*, 8(8), 589–594. Retrieved from https://doi.org/10.1007/BF00152152

Rani-Borges, B., Uemura Faria, A., de Campos, A., Costa Gonçalves, S. P., & Martins-Franchetti, S. M. (2016). Biodegradation of additive PHBV/PP-co-PE films buried in soil. *Polímeros: Ciência e Tecnologia*, 26(2), 161–167. Retrieved from https://www.redalyc.org/articulo.oa?id=47046375009

Rapisarda, M., Mistretta, M. C., Scopelliti, M., Leanza, M., La Mantia, F. P., & Rizzarelli, P. (2022). Influence of Calcium Carbonate Nanoparticles on the Soil Burial Degradation of Polybutyleneadipate-Co-Butylenetherephthalate Films. *Nanomaterials*, 12(13). Retrieved from https://doi.org/10.3390/nano12132275

Razzak, M. T., Darwis, D., Zainuddin, & Sukirno. (2001). Irradiation of polyvinyl alcohol and polyvinyl pyrrolidone blended hydrogel for wound dressing. *Radiation Physics and Chemistry*, 62(1), 107–113. Retrieved from https://doi.org/https://doi.org/10.1016/S0969-806X(01)00427-3

Rehman, R. (2015). Selection of potent bacterial strain for over-production of PHB by using low cost carbon source for eco-friendly bioplastics. *Advancements in Life Sciences*, 3, 29–35.

Rijk, T., Meer, P., Eggink, G., & Weusthuis, R. (2005). Methods for Analysis of Poly(3-hydroxyalkanoate) (PHA) Composition. In *Biopolymers Online* (Vol. 3). Retrieved from https://doi.org/10.1002/3527600035.bpol3b01

Rizzarelli, P., Cirica, M., Pastorelli, G., Puglisi, C., & Valenti, G. (2015). Aliphatic poly(ester amide)s from sebacic acid and aminoalcohols of different chain length: Synthesis, characterization and soil burial degradation. *Polymer Degradation and Stability*, 121, 90–99. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2015.08.010

Rocha, D. B., Souza de Carvalho, J., de Oliveira, S. A., & dos Santos Rosa, D. (2018). A new approach for flexible PBAT/PLA/CaCO3 films into agriculture. *Journal of Applied Polymer Science*, 135(35), 46660. Retrieved from https://doi.org/https://doi.org/10.1002/app.46660

Romo, D., Grosso, V., Moreno, N., & Castaño, D. (2007). A most effective method for selecting a broad range of short and medium-chain-length polyhidroxyalcanoate producing microorganisms. *Electronic Journal of Biotechnology*, 10, 348–357. Retrieved from https://doi.org/10.4067/S0717-34582007000300002

Roosen, M., Mys, N., Kusenberg, M., Billen, P., Dumoulin, A., Dewulf, J., ... De Meester, S. (2020). Detailed Analysis of the Composition of Selected Plastic Packaging Waste Products and Its Implications for Mechanical and Thermochemical Recycling. *Environmental Science & Technology*, 54(20), 13282–13293. Retrieved from https://doi.org/10.1021/acs.est.0c03371

Rudnik, E., & Briassoulis, D. (2011a). Comparative Biodegradation in Soil Behaviour of two Biodegradable

Polymers Based on Renewable Resources. *Journal of Polymers and the Environment*, 19(1), 18–39. Retrieved from https://doi.org/10.1007/s10924-010-0243-7

Rudnik, E., & Briassoulis, D. (2011b). Degradation behaviour of poly(lactic acid) films and fibres in soil under Mediterranean field conditions and laboratory simulations testing. *Industrial Crops and Products*, 33(3), 648–658. Retrieved from https://doi.org/https://doi.org/10.1016/j.indcrop.2010.12.031

Saadi, Z., Cesar, G., Bewa, H., & Benguigui, L. (2013). Fungal Degradation of Poly(Butylene Adipate-Co-Terephthalate) in Soil and in Compost. *Journal of Polymers and the Environment*, 21(4), 893–901. Retrieved from https://doi.org/10.1007/s10924-013-0582-2

Sabapathy, P. C., Devaraj, S., Anburajan, P., Parvez, A., Kathirvel, P., & Qi, X. (2023). Active polyhydroxybutyrate (PHB)/sugarcane bagasse fiber-based anti-microbial green composite: material characterization and degradation studies. *Applied Nanoscience*, 13(2), 1187–1199. Retrieved from https://doi.org/10.1007/s13204-021-01972-8

Sabapathy, P. C., Devaraj, S., Parthipan, A., & Kathirvel, P. (2019). Polyhydroxyalkanoate production from statistically optimized media using rice mill effluent as sustainable substrate with an analysis on the biopolymer's degradation potential. *International Journal of Biological Macromolecules*, 126, 977–986. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.01.003

Sadi, R., Fechine, G. J. M., & Demarquette, N. (2010). Photodegradation of poly(3-hydroxybutyrate). *Polymer Degradation and Stability*, 95, 2318–2327. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.09.003

Salguero, N. G., del Grosso, M. F., Durán, H., Peruzzo, P. J., Amalvy, J. I., Arbeitman, C. R., & García Bermúdez, G. (2012). Characterization of biodegradable polymers irradiated with swift heavy ions. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, 273, 47–50. Retrieved from https://doi.org/https://doi.org/10.1016/j.nimb.2011.07.035

Salim, Y. S., Sharon, A., Vigneswari, S., Mohamad Ibrahim, M. N., & Amirul, A. A. (2012). Environmental degradation of microbial polyhydroxyalkanoates and oil palm-based composites. *Applied Biochemistry and Biotechnology*, 167(2), 314–326. Retrieved from https://doi.org/10.1007/s12010-012-9688-6

Sang, B. I., Hori, K., Tanji, Y., & Unno, H. (2002). Fungal contribution to in situ biodegradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) film in soil. *Applied Microbiology and Biotechnology*, 58(2), 241–247. Retrieved from https://doi.org/10.1007/s00253-001-0884-5

Santhanam, A., & Sasidharan, S. (2010). Microbial production of polyhydroxy alkanotes (PHA) from Alcaligens spp. and Pseudomonas oleovorans using different carbon sources. *Afr J Biotechnol*, 9(21), 3144–3150. Retrieved from https://www.researchgate.net/publication/264850265_Microbial_production_of_polyhydroxy_alkanotes_PHA_from_Alcaligens_spp_and_Pseudomonas_oleovorans_using_different_carbon_sources

Sanyang, M. L., Ilyas, R. A., Sapuan, S. M., & Jumaidin, R. (2018). Sugar palm starch-based composites for packaging applications. *Bionanocomposites for Packaging Applications*, 125–147. Retrieved from https://doi.org/10.1007/978-3-319-67319-6_7

Sarath Kumara, P. H., Nagasawa, N., Yagi, T., & Tamada, M. (2008). Radiation-induced crosslinking and mechanical properties of blends of poly(lactic acid) and poly(butylene terephthalate-co-adipate). *Journal of Applied Polymer Science*, 109(5), 3321–3328. Retrieved from https://doi.org/https://doi.org/10.1002/app.28402

Scherer, T. M., Fuller, R. C., Lenz, R. W., & Goodwin, S. (1999). Production, Purification and Activity of an Extracellular Depolymerase from Aspergillus fumigatus. *Journal of Environmental Polymer Degradation*, 7(3), 117–125. Retrieved from https://doi.org/10.1023/A:1022881204565

Schick, C. (2009). Differential scanning calorimetry (DSC) of semicrystalline polymers. *Analytical and*

Bioanalytical Chemistry, 395(6), 1589–1611. Retrieved from https://doi.org/10.1007/s00216-009-3169-y

Schirmer, A., Matz, C., & Jendrossek, D. (1995). Substrate specificities of poly(hydroxyalkanoate)-degrading bacteria and active site studies on the extracellular poly(3-hydroxyoctanoic acid) depolymerase of Pseudomonas fluorescens GK13. *Canadian Journal of Microbiology*, 41(S1), 170–179. Retrieved from https://doi.org/10.1139/m95-184

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., ... Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, 109(16), 6241–6246. Retrieved from https://doi.org/10.1073/pnas.1117018109

Schröpfer, S., Karpinski Bottene, M., Bianchin, L., Robinson, L., Lima, V., Jahno, V., ... Ribeiro, S. (2015). Biodegradation evaluation of bacterial cellulose, vegetable cellulose and poly (3-hydroxybutyrate) in soil. *Polímeros*, 25, 154–160. Retrieved from https://doi.org/10.1590/0104-1428.1712

Šerá, J., Serbruyns, L., De Wilde, B., & Koutný, M. (2020). Accelerated biodegradation testing of slowly degradable polyesters in soil. *Polymer Degradation and Stability*, 171, 109031. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2019.109031

Šerá, J., Stloukal, P., Jančová, P., Verney, V., Pekařová, S., & Koutný, M. (2016). Accelerated Biodegradation of Agriculture Film Based on Aromatic-Aliphatic Copolyester in Soil under Mesophilic Conditions. *Journal of Agricultural and Food Chemistry*, 64(28), 5653–5661. Retrieved from https://doi.org/10.1021/acs.jafc.6b01786

Shah, A. A., Eguchi, T., Mayumi, D., Kato, S., Shintani, N., Kamini, N. R., & Nakajima-Kambe, T. (2013a). Purification and properties of novel aliphatic-aromatic co-polyesters degrading enzymes from newly isolated Roseateles depolymerans strain TB-87. *Polymer Degradation and Stability*, 98(2), 609–618. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.11.013

Shah, A. A., Hasan, F., & Hameed, A. (2010). Degradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a newly isolated Actinomadura sp. AF-555, from soil. *International Biodeterioration and Biodegradation*, 64(4), 281–285. Retrieved from https://doi.org/10.1016/j.ibiod.2009.10.012

Shah, A. A., Kato, S., Shintani, N., Kamini, N. R., & Nakajima-Kambe, T. (2014). Microbial degradation of aliphatic and aliphatic-aromatic co-polyesters. *Applied Microbiology and Biotechnology*, 98(8), 3437–3447. Retrieved from https://doi.org/10.1007/s00253-014-5558-1

Shah, Z., Hasan, F., Krumholz, L., Atkas, D., & Shah, A. A. (2013b). Degradation of polyester polyurethane by newly isolated Pseudomonas aeruginosa strain MZA-85 and analysis of degradation products by GC-MS. *International Biodeterioration and Biodegradation*, 77(April 2020), 114–122. Retrieved from https://doi.org/10.1016/j.ibiod.2012.11.009

Shang, L., Jiang, M., & Chang, H. N. (2003). Poly(3-hydroxybutyrate) synthesis in fed-batch culture of Ralstonia eutropha with phosphate limitation under different glucose concentrations. *Biotechnology Letters*, 25(17), 1415–1419. Retrieved from https://doi.org/10.1023/A:1025047410699

Shimao, M. (2001). Biodegradation of plastics. *Current Opinion in Biotechnology*, 12(3), 242–247. Retrieved from https://doi.org/https://doi.org/10.1016/S0958-1669(00)00206-8

Shin, D. Y., Park, J.-U., Choi, M.-H., Kim, S., Kim, H.-E., & Jeong, S.-H. (2020). Polydeoxyribonucleotide-delivering therapeutic hydrogel for diabetic wound healing. *Scientific Reports*, 10(1), 16811. Retrieved from https://doi.org/10.1038/s41598-020-74004-0

Shrivastav, A., Mishra, S., Pancha, I., Jain, D., Bhattacharya, S., Patel, S., & Mishra, S. (2011). Biodegradability studies of polyhydroxyalkanoate (PHA) film produced by a marine bacteria using Jatropha biodiesel byproduct as a substrate. *World Journal of Microbiology and Biotechnology*, 27. Retrieved from https://doi.org/10.1007/s11274-010-0605-2

Silva, I., Alves, M., Malheiro, C., Silva, A. R. R., Loureiro, S., Henriques, I., & González-Alcaraz, M. N.

(2022). Short-Term Responses of Soil Microbial Communities to Changes in Air Temperature, Soil Moisture and UV Radiation. *Genes*, 13(5). Retrieved from https://doi.org/10.3390/genes13050850

Sin, M. C., Gan, S. N., Annuar, M. S. M., & Tan, I. K. P. (2010). Thermodegradation of medium-chain-length poly(3-hydroxyalkanoates) produced by Pseudomonas putida from oleic acid. *Polymer Degradation and Stability*, 95(12), 2334–2342. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.08.027

Solomon, D. A. (2018). 5 - Integrating Molecular Diagnostics With Surgical Neuropathology. In A. Perry & D. J. B. T.-P. S. N. A. D. A. (Second E. Brat (Eds.) (pp. 71–89). Elsevier. Retrieved from https://doi.org/https://doi.org/10.1016/B978-0-323-44941-0.00005-9

Song, C., Wang, S., Ono, S., Zhang, B., Shimasaki, C., & Inoue, M. (2003). The biodegradation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/V) and PHB/V-degrading microorganisms in soil. *Polymers for Advanced Technologies*, 14, 184–188. Retrieved from https://doi.org/10.1002/pat.289

Song, J. H., Murphy, R. J., Narayan, R., & Davies, G. B. H. (2009). Biodegradable and compostable alternatives to conventional plastics. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 364(1526), 2127–2139. Retrieved from https://doi.org/10.1098/rstb.2008.0289

Soulenthone, P., Tachibana, Y., Muroi, F., Suzuki, M., & Ishii, N. (2020). Characterization of a mesophilic actinobacteria that degrades poly (butylene adipate- co -terephthalate). *Polymer Degradation and Stability*, 181, 109335. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2020.109335

Soulenthone, P., Tachibana, Y., Suzuki, M., Mizuno, T., Ohta, Y., & Kasuya, K. (2021). Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the mesophilic actinobacteria Rhodococcus fascians. *Polymer Degradation and Stability*, 184, 109481. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2021.109481

Sousa, F. M., Cavalcanti, F. B., Marinho, V. A. D., Morais, D. D. S., Almeida, T. G., & Carvalho, L. H. (2022). Effect of composition on permeability, mechanical properties and biodegradation of PBAT/PCL blends films. *Polymer Bulletin*, 79(7), 5327–5338. Retrieved from https://doi.org/10.1007/s00289-021-03745-3

Souza, P. M. S., Coelho, F. M., Sommaggio, L. R. D., Marin-Morales, M. A., & Morales, A. R. (2019). Disintegration and Biodegradation in Soil of PBAT Mulch Films: Influence of the Stabilization Systems Based on Carbon Black/Hindered Amine Light Stabilizer and Carbon Black/Vitamin E. *Journal of Polymers and the Environment*, 27(7), 1584–1594. Retrieved from https://doi.org/10.1007/s10924-019-01455-6

Spiekermann, P., Rehm, B. H., Kalscheuer, R., Baumeister, D., & Steinbuchel, A. (1999). A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Archives of Microbiology*, 171(2), 73–80. Retrieved from https://doi.org/10.1007/s002030050681

Sridewi, N., Bhubalan, K., & Sudesh, K. (2006). Degradation of commercially important polyhydroxyalkanoates in tropical mangrove ecosystem. *Polymer Degradation and Stability*, 91(12), 2931–2940. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2006.08.027

Steinbüchel, A., & Lütke-Eversloh, T. (2003). Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal*, 16(2), 81–96. Retrieved from https://doi.org/https://doi.org/10.1016/S1369-703X(03)00036-6

Strittmatter, C. S., Eggers, J., Biesgen, V., Hengsbach, J.-N., Sakatoku, A., Albrecht, D., … Steinbüchel, A. (2022). Insights into the Degradation of Medium-Chain-Length Dicarboxylic Acids in Cupriavidus

necator H16 Reveal β-Oxidation Differences between Dicarboxylic Acids and Fatty Acids. *Applied and Environmental Microbiology*, 88(2), e0187321. Retrieved from https://doi.org/10.1128/AEM.01873-21

Su, Z., Huang, S., Wang, Y., Ling, H., Yang, X., Jin, Y., ... Zhang, W. (2020). Robust, high-barrier, and fully recyclable cellulose-based plastic replacement enabled by a dynamic imine polymer. *Journal of Materials Chemistry A*, 8(28), 14082–14090.

Sudesh, K., Abe, H., & Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science*, 25(10), 1503–1555. Retrieved from https://doi.org/https://doi.org/10.1016/S0079-6700(00)00035-6

Sultanpuram, V., Mothe, T., & Mahmood, S. K. (2008). Biodegradation of polyhydroxyalkanoates. *The Internet Journal of Microbiology*, 4(2). Retrieved from https://www.researchgate.net/publication/284686017_Biodegradation_of_polyhydroxyalkanoates

Suyama, T., Tokiwa, Y., Ouichanpagdee, P., Kanagawa, T., & Kamagata, Y. (1998). Phylogenetic Affiliation of Soil Bacteria That Degrade Aliphatic Polyesters Available Commercially as Biodegradable Plastics. *Applied and Environmental Microbiology*, 64(12), 5008–5011. Retrieved from https://doi.org/10.1128/AEM.64.12.5008-5011.1998

Suzuki, K., Noguchi, M. T., Shinozaki, Y., & Koitabashi, M. (2014). Purification , characterization , and cloning of the gene for a biodegradable plastic-degrading enzyme from Paraphoma -related fungal strain B47-9, 4457–4465. Retrieved from https://doi.org/10.1007/s00253-013-5454-0

Tabasi, R. Y., & Ajji, A. (2015). Selective degradation of biodegradable blends in simulated laboratory composting. *Polymer Degradation and Stability*, 120(February), 435–442. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.07.020

Tachibana, Y., Kimura, S., & Kasuya, K.-I. (2015). Synthesis and Verification of Biobased Terephthalic Acid from Furfural. *Scientific Reports*, 5(8249). Retrieved from https://doi.org/10.1038/srep08249

Takahashi, K., Taniguchi, I., Miyamoto, M., & Kimura, Y. (2000). Melt/solid polycondensation of glycolic acid to obtain high-molecular-weight poly(glycolic acid). *Polymer*, 41(24), 8725–8728. Retrieved from https://doi.org/https://doi.org/10.1016/S0032-3861(00)00282-2

Tanaka, K., Katamune, K., & Ishizaki, A. (1995). Fermentative production of poly(β-hydroxybutyric acid) from xylose via L-lactate by a two-stage culture method employing Lactococcus lactis IO-1 and Alcaligenes eutrophus. *Canadian Journal of Microbiology*, 41(13), 257–261. Retrieved from https://doi.org/10.1139/m95-194

Tanase-Opedal, M., & Ruwoldt, J. (2022). Organosolv Lignin as a Green Sizing Agent for Thermoformed Pulp Products. *ACS Omega*, 7(50), 46583–46593. Retrieved from https://doi.org/10.1021/acsomega.2c05416

Tang, X., & Chen, E. Y.-X. (2018). Chemical synthesis of perfectly isotactic and high melting bacterial poly(3-hydroxybutyrate) from bio-sourced racemic cyclic diolide. *Nature Communications*, 9(1), 2345. Retrieved from https://doi.org/10.1038/s41467-018-04734-3

Tao, J., Song, C., Cao, M., Hu, D., Liu, L., Liu, N., & Wang, S. (2009). Thermal properties and degradability of poly(propylene carbonate)/poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PPC/PHBV) blends. *Polymer Degradation and Stability*, 94(4), 575–583. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2009.01.017

Teixeira, P. F., Covas, J. A., Suarez, M. J., Angulo, I., & Hilliou, L. (2020). Film Blowing of PHB-Based Systems for Home Compostable Food Packaging. *International Polymer Processing*, 35(5), 440–447. Retrieved from https://doi.org/10.3139/217.3985

Teramoto, N., Urata, K., Ozawa, K., & Shibata, M. (2004). Biodegradation of aliphatic polyester composites reinforced by abaca fiber. *Polymer Degradation and Stability*, 86(3), 401–409. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2004.04.026

Tezuka, Y., Ishii, N., Kasuya, K., & Mitomo, H. (2004). Degradation of poly(ethylene succinate) by mesophilic bacteria. *Polymer Degradation and Stability*, 84(1), 115–121. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2003.09.018

Thomas, S., Shumilova, A. A., Kiselev, E. G., Baranovsky, S. V., Vasiliev, A. D., Nemtsev, I. V., ... Volova, T. G. (2019). Thermal, mechanical and biodegradation studies of biofiller based poly-3-hydroxybutyrate biocomposites. *International Journal of Biological Macromolecules*, 155(15), 1373–1384. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.11.112

Thumarat, U., Kawabata, T., Nakajima, M., Nakajima, H., Sugiyama, A., Yazaki, K., ... Kawai, F. (2015). Comparison of genetic structures and biochemical properties of tandem cutinase-type polyesterases from Thermobifida alba AHK119. *Journal of Bioscience and Bioengineering*, 120(5), 491–497. Retrieved from https://doi.org/10.1016/j.jbiosc.2015.03.006

Thumarat, U., Nakamura, R., Kawabata, T., Suzuki, H., & Kawai, F. (2012). Biochemical and genetic analysis of a cutinase-type polyesterase from a thermophilic Thermobifida alba AHK119. *Applied Microbiology and Biotechnology*, 95(2), 419–430. Retrieved from https://doi.org/10.1007/s00253-011-3781-6

Toh, H. W., Toong, D. W. Y., Ng, J. C. K., Ow, V., Lu, S., Tan, L. P., ... Ang, H. Y. (2021). Polymer blends and polymer composites for cardiovascular implants. *European Polymer Journal*, 146, 110249. Retrieved from https://doi.org/https://doi.org/10.1016/j.eurpolymj.2020.110249

Trinh Tan, F., Cooper, D. G., Marić, M., & Nicell, J. A. (2008). Biodegradation of a synthetic co-polyester by aerobic mesophilic microorganisms. *Polymer Degradation and Stability*, 93(8), 1479–1485. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2008.05.005

Tripathi, S., Mehrotra, G. K., & Dutta, P. K. (2009). Physicochemical and bioactivity of cross-linked chitosan–PVA film for food packaging applications. *International Journal of Biological Macromolecules*, 45(4), 372–376. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2009.07.006

Tsuge, T., Yano, K., Imazu, S., Numata, K., Kikkawa, Y., Abe, H., ... Doi, Y. (2005). Biosynthesis of polyhydroxyalkanoate (PHA) copolymer from fructose using wild-type and laboratory-evolved PHA synthases. *Macromolecular Bioscience*, 5(2), 112–117. Retrieved from https://doi.org/10.1002/mabi.200400152

UNI 11462. (2012). Plastic Materials Biodegradable in Soil - Types, Requirements and Test Methods. *Italian Organization for Standardization*

Vagin, S., Winnacker, M., Kronast, A., Altenbuchner, P. T., Deglmann, P., Sinkel, C., ... Rieger, B. (2015). New Insights into the Ring-Opening Polymerization of β-Butyrolactone Catalyzed by Chromium(III) Salphen Complexes. *ChemCatChem*, 7(23), 3963–3971. Retrieved from https://doi.org/https://doi.org/10.1002/cctc.201500717

Vannini, C., Rossi, A., Vallerini, F., Menicagli, V., Seggiani, M., Cinelli, P., ... Balestri, E. (2021). Microbial communities of polyhydroxyalkanoate (PHA)-based biodegradable composites plastisphere and of surrounding environmental matrix: a comparison between marine (seabed) and coastal sediments (dune sand) over a long-time scale. *Science of the Total Environment*, 764, 142814. Retrieved from https://doi.org/10.1016/j.scitotenv.2020.142814

Varghese, S. A., Pulikkalparambil, H., Rangappa, S. M., Siengchin, S., & Parameswaranpillai, J. (2020). Novel biodegradable polymer films based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and Ceiba pentandra natural fibers for packaging applications. *Food Packaging and Shelf Life*, 25, 100538. Retrieved from https://doi.org/https://doi.org/10.1016/j.fpsl.2020.100538

Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology*, 102(6), 1437–1449. Retrieved from https://doi.org/10.1111/j.1365-2672.2007.03335.x

Volova, T. G., Prudnikova, S. V, Vinogradova, O. N., Syrvacheva, D. A., & Shishatskaya, E. I. (2017).

Microbial Degradation of Polyhydroxyalkanoates with Different Chemical Compositions and Their Biodegradability. *Microbial Ecology*, 73(2), 353–367. Retrieved from https://doi.org/10.1007/s00248-016-0852-3

Wallace, P. W., Haernvall, K., Ribitsch, D., Zitzenbacher, S., Schittmayer, M., Steinkellner, G., … Birner-Gruenberger, R. (2017). PpEst is a novel PBAT degrading polyesterase identified by proteomic screening of Pseudomonas pseudoalcaligenes. *Applied Microbiology and Biotechnology*, 101(6), 2291–2303. Retrieved from https://doi.org/10.1007/s00253-016-7992-8

Wang, H., Wei, D., Zheng, A., & Xiao, H. (2015). Soil burial biodegradation of antimicrobial biodegradable PBAT films. *Polymer Degradation and Stability*, 116, 14–22. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.03.007

Wang, J., Zheng, L., Li, C., Zhu, W., Zhang, D., Xiao, Y., & Guan, G. (2012). Fully biodegradable blends of poly(butylene succinate) and poly(butylene carbonate): Miscibility, thermal properties, crystallization behavior and mechanical properties. *Polymer Testing*, 31(1), 39–45. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2011.09.005

Wang, Q., Yu, H., Xia, Y., Kang, Z., & Qi, Q. (2009). Complete PHB mobilization in Escherichia coli enhances the stress tolerance: a potential biotechnological application. *Microbial Cell Factories*, 8(1), 47. Retrieved from https://doi.org/10.1186/1475-2859-8-47

Wang, Shichao, Chen, W., Xiang, H., Yang, J., Zhou, Z., & Zhu, M. (2016). Modification and potential application of short-chain-length polyhydroxyalkanoate (SCL-PHA). *Polymers*, 8(8). Retrieved from https://doi.org/10.3390/polym8080273

Wang, Shuai, Ma, P., Wang, R., Wang, S., Zhang, Y., & Zhang, Y. (2008). Mechanical, thermal and degradation properties of poly(d,l-lactide)/poly(hydroxybutyrate-co-hydroxyvalerate)/poly(ethylene glycol) blend. *Polymer Degradation and Stability*, 93(7), 1364–1369. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2008.03.026

Wang, Shufang, Song, C., Mizuno, W., Sano, M., Maki, M., Yang, C., … Takeuchi, S. (2005). Estimation on Biodegradability of Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/V) and Numbers of Aerobic PHB/V Degrading Microorganisms in Different Natural Environments. *Journal of Polymers and the Environment*, 13(1), 39–45. Retrieved from https://doi.org/10.1007/s10924-004-1214-7

Wang, X., Oehmen, A., Freitas, E. B., Carvalho, G., & Reis, M. A. M. (2017). The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production. *Water Research*, 112, 269–278. Retrieved from https://doi.org/10.1016/j.watres.2017.01.064

Wang, Y. Z., Zhou, Y., & Zylstra, G. J. (1995). Molecular analysis of isophthalate and terephthalate degradation by Comamonas testosteroni YZW-D. *Environmental Health Perspectives*, 103 Suppl(S5), 9–12. Retrieved from https://doi.org/10.1289/ehp.95103s49

Watanabe, T., Suzuki, K., Shinozaki, Y., & Yarimizu, T. (2015). A UV-induced mutant of Cryptococcus flavus GB-1 with increased production of a biodegradable plastic-degrading enzyme. *Process Biochemistry*, 50(11), 1718–1724. Retrieved from https://doi.org/10.1016/j.procbio.2015.07.005

Wei, D., Wang, H., Xiao, H., Zheng, A., & Yang, Y. (2015a). Morphology and mechanical properties of poly(butylene adipate-co-terephthalate)/potato starch blends in the presence of synthesized reactive compatibilizer or modified poly(butylene adipate-co-terephthalate). *Carbohydrate Polymers*, 123, 275–282. Retrieved from https://doi.org/10.1016/j.carbpol.2015.01.058

Wei, L., Liang, S., & McDonald, A. G. (2015b). Thermophysical properties and biodegradation behavior of green composites made from polyhydroxybutyrate and potato peel waste fermentation residue. *Industrial Crops and Products*, 69, 91–103. Retrieved from https://doi.org/https://doi.org/10.1016/j.indcrop.2015.02.011

Wen, X., & Lu, X. (2012). Microbial Degradation of Poly(3-Hydroxybutyrate-co-4-Hydroxybutyrate) in Soil. *Journal of Polymers and the Environment*, 20(2), 381–387. Retrieved from

https://doi.org/10.1007/s10924-011-0387-0

Weng, Y.-X., Wang, L., Zhang, M., Wang, X.-L., & Wang, Y.-Z. (2013). Biodegradation behavior of P(3HB,4HB)/PLA blends in real soil environments. *Polymer Testing*, 32(1), 60–70. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2012.09.014

Weng, Y.-X., Wang, Y., Wang, X.-L., & Wang, Y.-Z. (2010). Biodegradation behavior of PHBV films in a pilot-scale composting condition. *Polymer Testing*, 29(5), 579–587. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2010.04.002

Winnacker, M. (2019). Polyhydroxyalkanoates: Recent Advances in Their Synthesis and Applications. *European Journal of Lipid Science and Technology*, 121(11), 1900101. Retrieved from https://doi.org/https://doi.org/10.1002/ejlt.201900101

Witt, U., Einig, T., Yamamoto, M., Kleeberg, I., Deckwer, W. D., & Müller, R. J. (2001). Biodegradation of aliphatic-aromatic copolyesters: Evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. *Chemosphere*, 44(2), 289–299. Retrieved from https://doi.org/10.1016/S0045-6535(00)00162-4

Witt, Uwe, Müller, R.-J., & Deckwer, W.-D. (1995). New biodegradable polyester-copolymers from commodity chemicals with favorable use properties. *Journal of Environmental Polymer Degradation*, 3(4), 215–223. Retrieved from https://doi.org/10.1007/BF02068676

Witzler, M., Vermeeren, S., Kolevatov, R. O., Haddad, R., Gericke, M., Heinze, T., & Schulze, M. (2021). Evaluating Release Kinetics from Alginate Beads Coated with Polyelectrolyte Layers for Sustained Drug Delivery. *ACS Applied Bio Materials*, 4(9), 6719–6731. Retrieved from https://doi.org/10.1021/acsabm.1c00417

Woolnough, C. A., Yee, L. H., Charlton, T., & Foster, L. J. R. (2010). Environmental degradation and biofouling of 'green' plastics including short and medium chain length polyhydroxyalkanoates. *Polymer International*, 59(5), 658–667. Retrieved from https://doi.org/10.1002/pi.2746

Wu, C.-S. (2006). Assessing biodegradability and mechanical, thermal, and morphological properties of an acrylic acid-modified poly(3-hydroxybutyric acid)/wood flours biocomposite. *Journal of Applied Polymer Science*, 102(4), 3565–3574. Retrieved from https://doi.org/10.1002/app.24817

Wu, C.-S. (2014). Preparation and Characterization of Polyhydroxyalkanoate Bioplastic-Based Green Renewable Composites from Rice Husk. *Journal of Polymers and the Environment*, 22(3), 384–392. Retrieved from https://doi.org/10.1007/s10924-014-0662-y

Wu, C.-S., Liao, H.-T., & Cai, Y.-X. (2017). Characterisation, biodegradability and application of palm fibre-reinforced polyhydroxyalkanoate composites. *Polymer Degradation and Stability*, 140, 55–63. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.04.016

Wu, C. S. (2013). Preparation, characterization and biodegradability of crosslinked tea plant-fibre-reinforced polyhydroxyalkanoate composites. *Polymer Degradation and Stability*, 98, 1473–1480. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2013.04.013

Wu, C. S. (2016). Characterization and Biodegradation Evaluation of Biocapsules Composed of Polyester/Natural Product Composites. *Polymer - Plastics Technology and Engineering*, 55(4), 391–402. Retrieved from https://doi.org/10.1080/03602559.2015.1098683

Wu, S., Xiong, J., & Yu, Y. (2015). Taxonomic resolutions based on 18S rRNA genes: a case study of subclass copepoda. *PloS One*, 10(6), e0131498. Retrieved from https://doi.org/10.1371/journal.pone.0131498

Wufuer, R., Li, W., Wang, S., & Duo, J. (2022). Isolation and Degradation Characteristics of PBAT Film Degrading Bacteria. *International Journal of Environmental Research and Public Health*, 19(24). Retrieved from https://doi.org/10.3390/ijerph192417087

Xiao, B., Zheng, M., Pang, J., Jiang, Y., Wang, H., Sun, R., … Zhang, T. (2015). Synthesis and Characterization of Poly(ethylene terephthalate) from Biomass-Based Ethylene Glycol: Effects of

Miscellaneous Diols. *Industrial & Engineering Chemistry Research*, 54(22), 5862–5869. Retrieved from https://doi.org/10.1021/acs.iecr.5b00487

Xiong, S.-J., Pang, B., Zhou, S.-J., Li, M.-K., Yang, S., Wang, Y.-Y., ... Sun, R.-C. (2020). Economically Competitive Biodegradable PBAT/Lignin Composites: Effect of Lignin Methylation and Compatibilizer. *ACS Sustainable Chemistry & Engineering*, 8(13), 5338–5346. Retrieved from https://doi.org/10.1021/acssuschemeng.0c00789

Xu, J., & Guo, B.-H. (2010). Microbial Succinic Acid, Its Polymer Poly(butylene succinate), and Applications BT - Plastics from Bacteria: Natural Functions and Applications. In G. G.-Q. Chen (Ed.) (pp. 347–388). Berlin, Heidelberg: Springer Berlin Heidelberg. Retrieved from https://doi.org/10.1007/978-3-642-03287-5_14

Yamamoto, M., Witt, U., Skupin, G., Beimborn, D., & Müller, R.-J. (2005). Biodegradable Aliphatic-Aromatic Polyesters: "Ecoflex®". In *Biopolymers Online*. Retrieved from https://doi.org/https://doi.org/10.1002/3527600035.bpol4011

Yamane, T., Chen, X.-F., & Ueda, S. (1996). Polyhydroxyalkanoate synthesis from alcohols during the growth of Paracoccus denitrificans. *FEMS Microbiology Letters*, 135(2–3), 207–211. Retrieved from https://doi.org/10.1111/j.1574-6968.1996.tb07991.x

Yang, B., Wang, Y., & Qian, P.-Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*, 17(1), 135. Retrieved from https://doi.org/10.1186/s12859-016-0992-y

Yew, S.-P., Tang, H.-Y., & Sudesh, K. (2006). Photocatalytic activity and biodegradation of polyhydroxybutyrate films containing titanium dioxide. *Polymer Degradation and Stability*, 91(8), 1800–1807. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2005.11.011

Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., ... Van Dien, S. (2011). Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nature Chemical Biology*, 7(7), 445–452. Retrieved from https://doi.org/10.1038/nchembio.580

Zaidi, Z., Mawad, D., & Crosky, A. (2019). Soil Biodegradation of Unidirectional Polyhydroxybutyrate-Co-Valerate (PHBV) Biocomposites Toughened With Polybutylene-Adipate-Co-Terephthalate (PBAT) and Epoxidized Natural Rubber (ENR). *Frontiers in Materials*, 6(November), 1–12. Retrieved from https://www.frontiersin.org/article/10.3389/fmats.2019.00275

Zhang, M., Sharaf, F., & Chengtao, L. (2021). Screening and characterization of novel lipase producing Bacillus species from agricultural soil with high hydrolytic activity against PBAT poly (butylene adipate co terephthalate) co - polyesters. *Polymer Bulletin*, 79, 10053–10076. Retrieved from https://doi.org/10.1007/s00289-021-03992-4

Zhang, Y., Ma, J., O'Connor, P., & Zhu, Y.-G. (2022). Microbial communities on biodegradable plastics under different fertilization practices in farmland soil microcosms. *Science of The Total Environment*, 809, 152184. Retrieved from https://doi.org/10.1016/j.scitotenv.2021.152184

Zhao, G., Du, J., Chen, W., Pan, M., & Chen, D. (2019). Preparation and thermostability of cellulose nanocrystals and nanofibrils from two sources of biomass: rice straw and poplar wood . *Cellulose*, 26(16), 8625–8643. Retrieved from https://doi.org/10.1007/s10570-019-02683-8

Zhao, Q., Cheng, G., Song, C., Zeng, Y., Tao, J., & Zhang, L. (2006). Crystallization behavior and biodegradation of poly(3-hydroxybutyrate) and poly(ethylene glycol) multiblock copolymers. *Polymer Degradation and Stability*, 91(6), 1240–1246. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2005.09.003

Zhuang, C., Tao, F., & Cui, Y. (2015). Anti-degradation gelatin films crosslinked by active ester based on cellulose. *RSC Advances*, 5(64), 52183–52193. Retrieved from https://doi.org/10.1039/c5ra04808g

Zinn, M., & Hany, R. (2005). Tailored Material Properties of Polyhydroxyalkanoates through Biosynthesis and Chemical Modification. *Advanced Engineering Materials*, 7, 408–411. Retrieved from https://doi.org/10.1002/adem.200500053

Żołek-Tryznowska, Z., & Holica, J. (2020). Starch films as an environmentally friendly packaging material: Printing performance. *Journal of Cleaner Production*, 276, 124265. Retrieved from https://doi.org/https://doi.org/10.1016/j.jclepro.2020.124265

Zumstein, M. T., Rechsteiner, D., Roduner, N., Perz, V., Ribitsch, D., Guebitz, G. M., … Sander, M. (2017). Enzymatic Hydrolysis of Polyester Thin Films at the Nanoscale: Effects of Polyester Structure and Enzyme Active-Site Accessibility. *Environmental Science & Technology*, 51(13), 7476–7485. Retrieved from https://doi.org/10.1021/acs.est.7b01330

Zumstein, M. T., Schintlmeister, A., Nelson, T. F., Baumgartner, R., Woebken, D., Wagner, M., … Sander, M. (2018). Biodegradation of synthetic polymers in soils: Tracking carbon into CO2 and microbial biomass. *Science Advances*, 4(7). Retrieved from https://doi.org/10.1126/sciadv.aas9024

CHAPTER 3.

BIODEGRADATION OF PHBV/PBAT FILMS IN SOIL

3.1 INTRODUCTION

The PHA family shows great potential to be implemented as the best alternative to conventional plastics since several types of bacteria and fungi are capable of decomposing them (Boyandin et al., 2012a; Sang et al., 2002). The copolymer polyhydroxybutyrate-co-hydroxyvalerate (PHBV) is considered a biodegradable material in soil and usually presents a biodegradation above 90 % with a fast degradation rate (Lammi et al., 2019); even when the biodegradation requirement of the control (> 70 %, in 180 days) had not been reached, the PHBV still reaches considerable values (Palsikowski et al., 2018). This trend is also maintained whenever weight loss is used to analyse biodegradation (Gonçalves et al., 2009; Thomas et al., 2019). However, although it has interesting properties, it is much more expensive than conventional plastics, which limits its wide use and application (Lammi et al., 2019).

Blending PHA with other polymers, such as polybutylene adipate terephthalate (PBAT) has been explored recently (Javadi et al., 2010; Larsson et al., 2016). PBAT has attracted extensive attention from researchers due to its properties, including its ductility, flexibility, and biocompatibility (Fukushima et al., 2012; Kijchavengkul et al., 2010a; Nagarajan et al., 2013; Nar et al., 2014; Pal et al., 2020). The characteristics of PBAT make it a potential candidate for an extensive range of applications such as agricultural and packaging films to medical devices (Fukushima et al., 2012). However, PBAT is only considered compostable (Witt et al., 2001). It is considered resistant to biodegradation in natural environments such as soil (Han et al., 2021). Polyesters such as PBAT are insoluble polymers, which indicates that they cannot be directly absorbed by microorganisms. For the polymer to be depolymerized and possibly used, it must be hydrolysed outside the microorganisms, using for example enzymes (Wallace et al., 2017). The degradation of PBAT is considered slow, especially at mesophilic temperature, nevertheless in real soil is significantly faster (Nikolić et al., 2017). This type of outcome indicates the great potential for PBAT to be used in several applications, including food packaging applications. Nonetheless, recently PBAT films prepared using 4 different methods, presented differences in physical, mechanical, and structural properties including the degree of crystallinity (Li et al., 2015). These types of differences may cause different biodegradation results and must be taken into consideration. Blends or bilayers of PHBV and PBAT were successfully developed to be applied in the packaging sector (Cunha et al., 2016; Russo et al., 2013). However, these works do not analyse the biodegradation of the materials.

Even though these two polymers are considered biodegradable or compostable, little is known about their biodegradability by soil microbiomes. Plastics are more likely to end up in mesophilic conditions rather than in compost at relatively high temperatures, so it is of great importance to study the behaviour of

biodegradable materials at ambient temperature (Kasuya et al., 2009). Since most plastics end up in landfills and soil, it is crucial to evaluate the biodegradability of these plastic blends in soil, to evaluate the impact of their utilization. Advances in material development are far ahead of an understanding of lifetime analyses of emerging blends/composite materials. PHBV/PBAT bilayer films produced for food packaging were analysed in terms of their characteristics such as rheological properties, and the systematic study of the impact of the processing parameters on the properties of the film was also evaluated (Cunha et al., 2016). The objective of this work was to investigate the biodegradation of this PHBV/PBAT film in soil using the carbon dioxide (CO_2) evolution and to confirm changes in the film physiochemical properties by scanning electron microscopy (SEM), attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA). ATR-FTIR spectroscopy is an efficient technique that has been used in several works, including in the analysis of the differences in the structure of polymer samples through the composting process (Tabasi and Ajji, 2015). It has been also used to analyse the effects of the biodegradation in soil of polymers (Palsikowski et al., 2018; Weng et al., 2013a). TGA is a reliable technique that can be used to analyse the thermal stability and decomposition of polymeric materials. It determines the rate of change in weight of the material, while the material is being heated at a controlled temperature (Sin et al., 2010). The microbiology of the process was also investigated by performing a shotgun taxonomic analysis of the soil microbial communities, before and after the biodegradation process. In addition, microbial isolates with the ability to biodegrade the film were obtained and identified.

3.2 MATERIAL AND METHODS

3.2.1 Soil media

The soil sample was collected at the University of Minho (Campus of Gualtar, Braga, Portugal) according to ISO 18400 (2018). Since aerobic agricultural soil was required, sampling was conducted from the surface to the actual ploughing depth, and the vegetation encountered, or other visible sources of organic carbon that could hide the CO_2 produced from the tested material incubations was excluded. The soil was sampled in June 2020, with normal temperatures and conditions for the season (ranging from 12 °C to 30 °C). The soil was transported in a loosely tied black polyethylene bag to be kept in the dark with free access to air, to avoid exposure to light that could encourage the growth of algae on the surface of the soil, physical compaction, and reduce variations in the soil water content as recommended by the ISO

18400 (2018). The soil was sieved to less than 2 mm to fulfil the demand of the ASTM D5988-18 (2018) and then stored in the dark at (4 ± 2) °C in trays with 2 cm of height to maintain the aerobic conditions.

The physicochemical characteristics of the soil were determined using the recommended guidelines in the ASTM D5988-18 (2018) namely the ASTM D1293-18 (2018), ASTM D425-17 (2017) and SM 2540 G (1998) for pH, moisture-holding capacity and the content of total, volatile and fixed solids, respectively. the carbon to nitrogen ratio was determined by elemental analysis using a LECO TruSpec CHN.

3.2.2 Biodegradation experiments set-up

The biodegradation tests were performed at 27 ± 0.5 °C using 9 reactors in parallel. The soil (300 g) was placed in a 3 L wide-mouth glass jar (reactor), as represented in Figure 3.1.

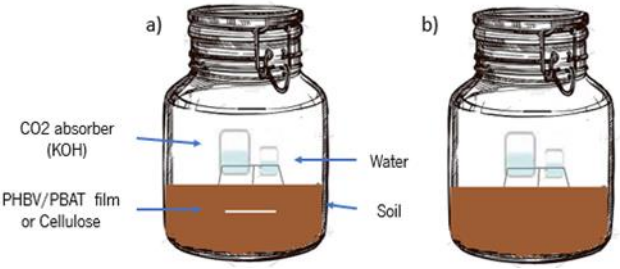

Figure 3.1 Biodegradation test set-up: a) Reactor with PHBV/PBAT film or cellulose, b) Control reactor with no film.

The test specimens, PHBV/PBAT 70/30 % in weight bilayer film (12 X 8.5 cm with 121 microns of thickness) were placed in 3 reactors, and 3 control assays were prepared as recommended by the ASTM D5988-18 (2018), using cellulose paper (Whatman no. 1 (8 X 12 cm with 197 microns of thickness)) as carbon and energy source. In the last 3 bioreactors, no carbon source was added (blank bioreactors). Distilled water was added to bring the moisture to 90 % of the moisture-holding capacity. The biodegradation was assessed by respirometric tests, following the ASTM D5988-18 (2018) test method using the measurement of CO_2 production by titration.

The titration method to measure the CO_2 was accomplished accordingly to the ASTM D5988-18 (2018) with the following differences: jars were used instead of desiccators, and 0.5 $mol.L^{-1}$ potassium hydroxide (KOH) solution (Labkem) was placed in a 150 mL beaker instead of a 100 mL beaker. An automatic titrator Titrando 888, with the Tiamo™ 2.5 software, was used to perform the titration with hydrochloric acid (HCl) solution 0.25 $mol.L^{-1}$ (Labbox AGR ISO). When necessary (during KOH replacement), distilled water was added to the soil to conserve the initial moisture-holding capacity.

Briefly, the evolved CO_2 was captured by the solution, according to the following reaction:

$$2\,\mathrm{KOH} + CO_2 \rightarrow \mathrm{K_2CO_3} + \mathrm{H_2O}$$

The potassium hydroxide reacts with the CO_2 produced, creating potassium bicarbonate. Then the quantity of CO_2 produced was determined by titrating the KOH solution with HCL to a phenolphthalein end-point The net CO_2 produced from the test material was calculated by subtracting the average amount of CO_2 produced in the soil control jars from the amount of CO_2 produced in the test material jars. The biodegradation percentages were calculated from the ratio between the net CO_2 production and the theoretical CO_2 production based on the carbon content, of the tested material.

The blown film was a PHBV/PBAT 70/30 % in weight bilayer material. The details about the production of the film can be found elsewhere (Cunha et al., 2016). The commercial PHBV with 3 % of 3-hydroxyvalerate (ENMAT TM Y1000P, produced by Tianan Biologic Materials Company, with a density of 1.25 g/cm^3 and T_g of 8 °C and T_m of 165 °C) contained 3 mol % HV, and the PBAT was a commercial aliphatic-aromatic copolyester-based polymer (EcoflexVR F blend C1200, a film blowing grade. The cellulose's carbon content is 44.4 % of its weight, taking into account its chemical composition $(C_6H_{10}O_5)_n$. The carbon content of the film was determined by elemental analysis, and the data is presented in Table 3.1.

Table 3.1 Weight and total carbon content of the materials tested. All experiments were performed in triplicate

Bioreactor	Test Material	Carbon (%)	Total Amount of test material (mg)	Total carbon of test Material (mg)
PHBV/PBAT Film	PHBV (70 %) PBAT (30 %)	62..7	1000.0 ± 0.1	627.0 ± 0.1
Cellulose filter paper	Cellulose (100 %)	44.4	1001.3 ± 0.5	445.0 ± 0.2

Samples were taken at the beginning and end of the incubations for determination of the microbial diversity. For that, soil samples were preserved in phosphate-buffered saline solution at -20 °C, till DNA extraction. The films were analysed before and after the incubations by SEM, ATR-FTIR, DSC and TGA to verify the effects on the surface, to analyse the functional groups of the films and to evaluate the changes in the thermal properties, respectively.

3.2.3 Analytical and microscopic methods

Selected test films were analysed using a desktop SEM (Phenom ProX, Netherlands) accordingly to Ferreira-Santos et al., (2021). All results were acquired using the ProSuite software. The samples were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 2 nm of gold (Au) for improved conductivity. The aluminium pin stub was then placed inside a Phenom Standard Sample Holder. The image analysis was conducted at 5 kV (intensity). For ATR-FTIR analysis an ALPHA II - Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance cell was used, with the method described by Silva et al. (2022) but in the wavenumber region of 400–4000 cm^{-1}. A total of 64 scans were performed for each sample, with a resolution of 4 cm^{-1}. Six spectra were recorded from each side of the sample film. Analyses were conducted using dried samples.

The films were analysed with a Perkin Elmer DSC 4000 Differential Scanning Calorimeter (Perkin Elmer, Waltham, MA, USA) based on the analysis of (Silva et al., 2021). Film samples (8 mg each) were placed into the DSC aluminium pan (B0143016) before measurement. An empty pan was used as a reference. The films were heated from 5 °C to 190 °C at 10 °C/min to erase the thermal history, then cooled down to -25 °C at -10 °C/min under nitrogen atmosphere, and finally reheated to 190 °C at 10 °C/min. The final heating run was used to detect the melting and crystallization temperatures (T_m, T_c), the onset temperatures (T_m Onset) and melting enthalpies (ΔH) calculated using Pyris software version 12.1 (Perkin Elmer, Waltham, MA, USA). The crystallinity (X_c) of the samples was determined by the ratio of the melting enthalpy (ΔH_0) for 100 % crystalline PHBV or PBAT, which is 146 J/g (Rosa et al., 2004) or 114 J/g, respectively (Chivrac et al., 2006), as described by Beber et al. (2018). The absolute crystallinity can be calculated by:

$$X_c = \frac{\Delta H}{\Delta H0} \times 100$$

Where ΔH is the enthalpy of fusion (J/g) of each polymer in the bilayer.

The same equipment and program were also used according to Silva et al. (2022) for TGA analysis. Samples (20 mg) were placed and weighed in an alumina crucible on the equipment's scale. Analysis was conducted from 25 to 600 °C at 10 °C/min under a nitrogen atmosphere. The weight loss, in percentage, and its derivative were represented as a function of temperature. The decomposition peak temperature (T_d) was determined by observing the peak in the weight loss slope of the derivative. The onset temperature of decomposition (T_d onset) was also determined.

3.2.4 Agar plate containing plastic

The growth medium consisted of a mineral base medium with no other source of carbon aside from the polymers themselves (Malik, 1988). Briefly, for dispersion (Solution A), 2 g of PHBV powder was added to 92 mL of distilled water and placed for 30 min in the ultrasound bath followed by 30 min of magnetic stirring. This procedure was performed twice. In another beaker, 7.5 g of agar was dispersed and melted in 368 mL of distilled water. Then, a Pasteur pipette was used to dropwise the PHBV dispersion to the melted agar under moderate and constant stirring. This dropwise approach has been used elsewhere (Charnock, 2021). Additionally, Solution B (2.3 g KH_2PO_4 and 2.9 g $Na_2HPO_4.2H_2O$ prepared in 50 mL of distilled water), Solution C (1 g NH_4Cl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.005 g $MnCl_2.4H_2O$), 5 mL of a trace element solution and 7.5 g of agar in 460 mL of distilled water), and Solution D (0.05 g ferric ammonium citrate in 20 mL distilled water) were prepared (Malik, 1988). All solutions were autoclaved separately, cooled down to 60 °C, mixed aseptically with 10 mL vitamin solution (filter sterilized) and about 15 mL was poured into 90 mm Petri plate dishes. The final pH was 6.8 without adjustment. The vitamin solution was composed of 10 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg pyridoxine HCl, 50 mg calcium pantothenate, 0.1 mg biotin, 0.2 mg folic acid, 1 mg B12 in 200 mL distilled water (Malik, 1988). The trace element stock solution had 0.1 g $ZnSO_4.7H_2O$, 0.03 g $MnCl_2.4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2.6H_2O$, 0.01 g $CuCl_2.2H_2O$, 0.02 g $NiCl_2.6H_2O$ and 0.03 g $Na_2MoO_4.2H_2O$ for 1 L distilled water (Malik, 1988).

Concerning the PBAT plates, the same proportion of nutrients, vitamins, and agar was used. However, instead of direct incorporation in the medium, small film rectangles that had previously been exposed to 1 h of UV light (both sides) were placed on top of the freshly done plates to ensure good adhesion to the surface (Rose et al., 2020; Urbanek et al., 2017).

3.2.5 Isolation of microorganisms degrading the polymer film

At the end of the incubations, the 3 films were recovered from soil (from the 3 reactors) using sterilized tweezers and washed with 50 mL phosphate-buffered saline solution. The resulting phosphate-buffered saline solution containing soil and soil microorganisms of the 3 containers was mixed and submitted to serial dilutions (from 10^{-1} to 10^{-9}). An aliquot of 0.1 mL (of each dilution) was plated in agar plates (in duplicate), containing mineral medium and PHB or PBAT as the sole carbon source, and incubated at 27 °C. In the case of PHBV, plates were examined for the development of clearing zones around colonies. Only different colony types evaluated visually based on colour, shape, pattern, and consistency were selected for further isolation. After 5 days of growth, these colonies were transferred to the same medium.

The taxonomic identity of the isolates was obtained by sequencing the 16S rRNA genes coding for the small subunit of the ribosome, in the case of *Bacteria*, or the internal transcribed spacer (ITS) between the small and the large subunit of the ribosome, in the case of *Fungi*.

3.2.6 DNA isolation, polymerase chain reaction (PCR) and Sanger and Illumina sequencing

DNA extraction was performed with the FastDNA Spin kit for soil (MP Biomedicals, Solon, OH), following the manufacturer's instructions. The quality and concentration of DNA were determined by using a Nanodrop® 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For the identification of bacterial isolates, the 16S rRNA gene was amplified by PCR, using the universal primers Bact27F (GTTTGATCCTGGCTCAG) and Uni1492R (CGGCTACCTTGTTACGAC), and for *Fungi* identification, the ITS was amplified with primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (Lane, 1991; White et al., 1990).

PCR programs can be found elsewhere, Salvador et al. (2019) and Santos et al (2020). The Taq polymerase used was MyTaq™ and the reaction volumes were used as indicated in the enzyme instruction kit. The size and yield of PCR products were estimated using the GeneRuler 1 kb Plus DNA ladder (Life Technologies, UK) in gel electrophoresis using agarose gel (1 % wt/vol) stained with GreenSafe Premium (NZYTech, Lisbon, Portugal). The samples were sequenced using the Sanger method at Macrogen (Spain). Sequences were aligned using Bioedit program version 7.2.5 (Hall, 1999) and the consensus region of overlap was used to compare with homologous sequences in the NCBI database by local alignment using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) to obtain the taxonomic identification of the isolates.

The diversity of the soil microbial community was determined through the isolation of total DNA from the soil sample of the inoculum and at the end of the trials (sampling and sequencing were done in triplicate). The samples were sequenced in an Illumina MiSeq platform at RTL Genomics (Lubbock, Texas), where amplification with Illumina and specific primers, library preparation, sequencing and bioinformatics data analysis were performed. Details on the method are given elsewhere (Salvador et al., 2019). The primer sets used for sequencing were: 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) for the prokaryotic community, targeting the 16S rRNA gene, and EUK1391F (GTACACACCGCCCGTC) and EUKBR (TGATCCTTCTGCAGGTTCACCTAC) for the eukaryotic community, targeting the 18S rRNA gene (Caporaso et al., 2011; Lane, 1991; Medlin et al., 1988). All FASTA files obtained from Sanger sequencing (corresponding to the microbial isolates) and FASTQ files

obtained from Illumina sequencing (corresponding to the taxonomic analysis of soil microbial communities) have been submitted to ENA under the accession numbers PRJEB62455 and PRJEB60413, respectively. The accession numbers of the ITS and 16S rRNA sequences obtained via Sanger sequencing were ERZ21821879 and ERZ21821878 respectively.

3.2.7 Statistical analysis

All the DSC and TGA values were expressed as mean ± standard deviation (SD) for the initial and final PHB/PBAT films before and after degradation. The data was assessed using a one-way analysis of variance (ANOVA) followed by Tukey´s test using the OriginPRO 2019b statistical program and the statistical significance was accepted as $p < 0.05$ (95 % significance). The analysis of the microorganisms' relative abundance obtained before and after PHBV/PBAT incubation from the Illumina sequencing was performed using Multiple t-tests followed by Holm-Sidak (statistical significance: $p < 0.05$) with the program GraphPad Prism version 8.0.1.

3.3 RESULTS

3.3.1 Biodegradation assays

The ASTM D5988-18 (2018) requires the analysis of the physicochemical properties of the soil. The pH must be between 6-8 and as can be observed in Table 3.2 this condition was fulfilled.

Table 3.2 Physicochemical properties of the soil. All experiments were performed in triplicate

Soil Parameters	
Total dry solids[1] (%)	94.23
Volatile solids[1](%)	8.07
pH[2]	6.30
Total organic carbon amount[3] (%)	2.82
Total nitrogen amount[3] (%)	0.30
Carbon/nitrogen ratio[3] (C:N)	9.40
Moisture holding capacity[4] (%)	19.00

*1 –Determined using the SM 2540 G (1998), *2 – Determined using the ASTM D1293 (2018), *3 –Determined by elemental analysis, *4 – Determined using the ASTM D425 (2017)

The visual appearance of the surfaces of the material at the beginning and end of the test is presented in Figure 3.2. All samples darkened throughout the burial period due to the soil attachment on the samples' surfaces. After 7 months, the material presented obvious signs of biodegradation and clear damage from

both sides, some pieces showed a red coloration. These signs of discoloration are expected in soil environments due to microbial attack.

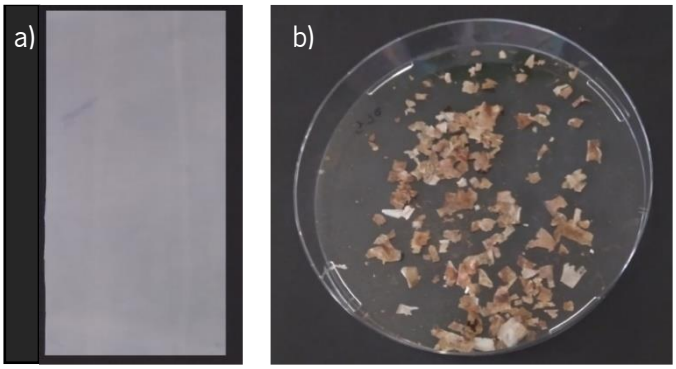

Figure 3.2 a) PHBV/PBAT film. b) Degradation residues after 7 months of degradation.

The cumulative CO_2 progress of the different materials incubated at 27 °C is shown in Figure 3.3a. The control released more CO_2 than the films because it was degraded faster (3.27 mg of CO_2/mg of carbon after 220 days) than the bilayer film (1.69 mg of CO_2/mg of carbon after 220 days).

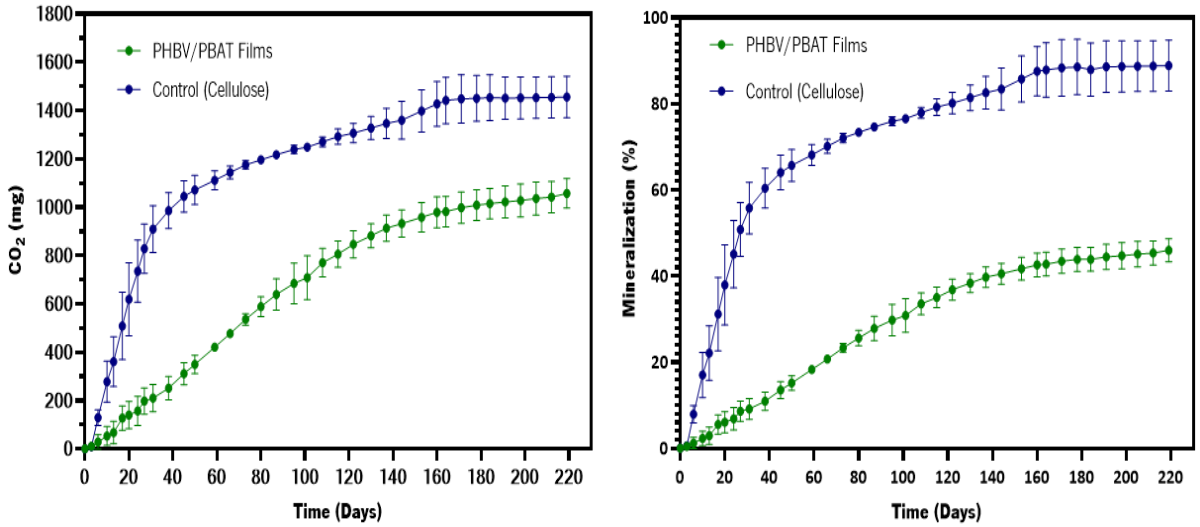

Figure 3.3 a) Carbon dioxide evolution for the different materials. b) Carbon mineralization curves of the tested materials, PHBV/PBAT films and cellulose (control material) by soil microbiomes. All experiments were performed in triplicate.

The mineralization curves for the studied samples are represented in Figure 3.3b. The reference material, cellulose, presented the highest mineralization rate, which is consistent with its use as a positive control. For the assay to be valid, at least 70 % of the reference material must be degraded in the first 6 months (ASTM D5988-18, 2018), and that condition was met after 2 months and 6 days when about 20 % of the

PHBV/PBAT have been degraded. This indicates a proper number of decomposing microorganisms with the capacity to degrade polymers.

The PHBV/PBAT samples reached the end of the assay (more than 7 months) with 46.0 ± 2.7 % of biodegradation and the control with an average of 88.9 ± 5.9 % in 220 days with the soil microorganisms as the main responsible for this result. The control had a fast biodegradation rate in the first 2 months especially in the first 30 days (1.8 %/day), then between days 30 to 160 the rate diminished to about 0.2 %/day, but in the last 60 days, it only degraded an average of 1.5 % of cellulose. Concerning the PHBV/PBAT biodegradation rate, it was initially slower than the cellulose degradation, but remarkably, no lag phase was observed since oxygen consumption began at the beginning of the incubations, showing that the microbial community could biodegrade the plastic film. A considerable mineralization of the film was observed after 4 months of incubation (35.3 %), and thereafter the biodegradation rate slowed down (rate 0.3 %/day) till the end of the assay.

3.3.2 Analytical and microscopic results

The surface of the buried samples was examined by conducting SEM, to identify signs of microorganism activity. Figures 3.4a and 3.4b show SEM micrographs of the film surfaces, from both sides, at the beginning of the assays. Figure 3.4 (c, d, e and f) correspond to the films after 7 months of soil burial. The microbial colonization and attack on the surfaces of the films were clearly visible, with the presence of various cracks and surface erosion (Figure 3.4e). In Figure 3.4d it is possible to see a network of fungal hyphae filaments embedded within the matrix along with the presence of spores that can be seen in Figures 3.4e and 3.4f. This suggests that *Fungi* may had a fundamental role in the biodegradation of the bilayer film.

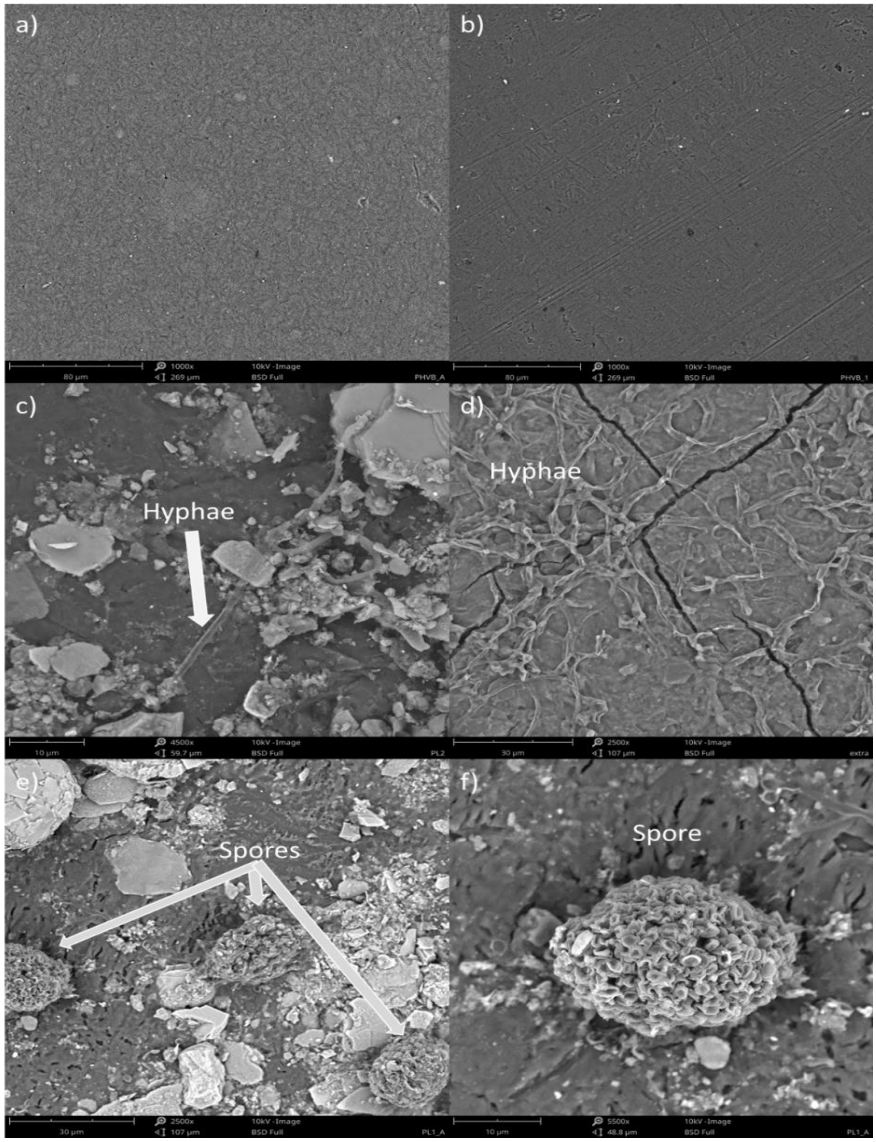

Figure 3.4 Scanning electron micrographs of plastic films after 7 months incubation in soil. a) PHBV layer before incubation, b) PBAT layer before incubation, c) PBAT layer after incubation, evidencing the presence of hyphae, e) PBAT layer after incubation, evidencing the presence of spores, d) PHBV layer after incubation, evidencing the presence of hyphae and f) PBAT layer after incubation, evidencing the presence of spores.

The ATR-FTIR of the initial PHBV side presented the typical absorption peaks (Table 3.3), including the 1718 cm^{-1} corresponding to C=O cm^{-1}, 1273 cm^{-1} to C–O, 1379 cm^{-1} to CH_3, 1453 cm^{-1} to CH_2 and 2931 cm^{-1} to C-H between others (Reddy et al., 2009).

Table 3.3 Infrared spectral analysis of the samples before and after degradation

Wavenumber (cm^{-1})			
PHBV side		**PBAT side**	
Before Biodegradation	**After Biodegradation**	**Before Biodegradation**	**After Biodegradation**
3435		.	3694
2975			3618
2931		3411	3436
1718		2955	2951
1453		1709	1708
1402		1504	1504
1379		1458	1457
1358		1409	1409
1273		1391	1390
1260		1367	1367
1225		1321	1321
1179		1269	1270
1129		1251	1251
1099		1207	1207
1053		1161	1162
1043		1118	1117
977		1103	1102
953		1017	1016
937		932	933
910		873	873
894		796	796
838		751	751
825		726	725
678		589	533
623		497	496
514			467
459			428
429			

As indicated in Table 3.3, the typical spectrum of the PBAT is present in the initial sample. For example, the presence of 2950 cm^{-1} peak corresponds to the C-H stretching, 1708/1709 cm^{-1} corresponds to the stretching vibration of C=O, 1504 cm^{-1} represents the skeleton vibration of the benzene ring, 1409 cm^{-1} and 1391 cm^{-1} correspond to C-H in-plane deformation vibration and C-H wagging vibration, 1269 cm^{-1}

corresponds to the symmetric stretching vibration of C–O, 1103 cm^{-1} represents the C-O-C left-right symmetric stretching vibration absorption, 1017 cm^{-1} represents the C-H in-plane deformation vibration of two adjacent surfaces on the *p*-substituted benzene ring, 931 cm^{-1} represents the trans-C–O symmetric stretching vibration and 725 cm^{-1} represents the bending vibration absorption of C-H off-plane of the benzene ring (Kijchavengkul et al., 2010a; Qi et al., 2021; Weng et al., 2013b). After incubation, only PBAT spectra were found, indicating complete or nearly complete degradation of the PHBV film (Table 3.3). Some of these peaks had slight differences in the positions after the assay which may indicate biodegradation. However, those differences are within the error margin (4 cm^{-1}) so they should not be valued. The peak corresponding to the C-H stretching changed from 2954 to 2950 cm^{-1} and a reduction of the absorbance peak was also observed. Nonetheless, all peaks apart from the 1017 cm^{-1} and 931 cm^{-1} had marked reductions after biodegradation including the carbonyl absorbance (1708 cm^{-1}).

Based on the TGA analysis it was possible to identify two clear stages in the initial film, between 263.0 – 312.3 °C and 351.0 – 430.3 °C, which corresponded to the PHBV and PBAT fractions, respectively. Neat PBAT film normally presents a decomposition temperature (T_d) of around 400 °C (Li et al., 2015; Qi et al., 2021). On the other hand, PHBV presents T_d slightly below 300 °C (Arcos-Hernandez et al., 2012). These 2 stages have also been observed in other blends of PHB with PBAT (Larsson et al., 2016). After more than 7 months of biodegradation only 1 stage (Table 3.4)., the PBAT stage (351.0 °C – 430.3 °C), was detected.

Table 3.4 Thermal properties of the bilayer film before and after incubation in soil, determined by TGA. All experiments were performed in triplicate

Samples	T_{d^1} (°C)	Speed max¹	T_d onset¹ (°C)	weight loss¹ (%)	T_{d^2} (°C)	Speed max²	T_d onset² (°C)	weight loss² (%)
Initial PHBV/PBAT Film	293.5 ± 2.3	13.0 ± 13	277.5 ± 3.6	29.7 ± 5.9	409.1 ± 5.3	14.5 ± 1.1	372.7 ± 4.9	54.3 ± 4.6
Final PHBV/PBAT Film					403.7 ± 0.7	17.3 ± 1.5	380.2 ± 0.5	64.1 ± 4.6

The onset which is the temperature at which the weight loss starts to be significant in the PBAT stage increased from 372.7 °C to 380.2 °C. Concerning weight loss, the initial samples had an average loss of 29.6 % and 54.3 % for the corresponding two stages. The Td, determined at the maximum weight loss rate of the samples decreased from 409.1 °C to 403.7 °C. The final samples presented a superior

average weight loss (63.3 %) which can be explained by the presence of a highest PBAT in this final sample, due to the PHBV biodegradation (Table 3.4).

The DSC analyses also confirmed that the PHBV was not detected in all samples after incubation. In the initial sample, two distinctive peaks at 126 °C and 169 °C were clear, corresponding to PBAT and PHBV, respectively. These values were very similar to others, where blended PHBV and PBAT were produced (Pal et al., 2020). In the final sample, the peak corresponding to the PHBV disappeared from all samples. The PBAT peak was maintained, and each of the measured parameters was similar before and after the assay. The crystallinity of the bilayer decreased after incubation, which is due to the biodegradation of PHBV, and thus its absence in the final film. Considering the portion corresponding only to the PBAT layer, the crystallization has increased, which is confirmed by a broader peak and the values presented in Table 3.5. This result indicates the preference of the microorganisms for the amorphous regions within PBAT.

Table 3.5 Thermal properties of the samples before and after degradation determined by DSC. All experiments were performed in triplicate

Samples	T_m^1 (°C)	ΔH^1 (J/g)	T_m Onset^1 (°C)	T_c^1 (°C)	X_c^1 (%)	T_m^2 (°C)	ΔH^2 (J/g)	T_m Onset^2 (°C)	T_c^2 (°C)	X_c^2 (%)
Initial PHBV/PBAT Film	169.4 ± 0.6	23.0 ± 3.2	164.5 ± 0.4	115 ± 1.9	15.8 ± 2.2	126.0 ± 2.4	3.6 ± 0.5	108.3 ± 0.3	87.0 ± 0.6	3.1 ± 0.4
Final PHBV/PBAT Film						126.4 ± 0.8	10.9 ± 2.4	106.3 ± 2.0	91.2 ± 0.7	9.5 ± 2.1

The increased crystallinity revealed that the amorphous regions of these polymers were degraded at higher rates (Table 3.5). The thermal decomposition analysis revealed that the thermal stability of the films decreased over time, and some physical changes occurred in the bilayer film structure due to the biodegradation in the soil.

The results of the CO_2 analysis and the disappearance of PHBV show that all the PHBV has been enzymatically cleaved into oligomers, dimers, and monomers. However, not all these low molecular products have been mineralized into CO_2, because only 45 % of the film has been mineralized and PHBV corresponded to 70 % of its composition.

3.3.3 Diversity of soil microbial communities before and after the incubation

The diversity analysis revealed that microorganisms assigned to the domain *Bacteria* were dominant in the prokaryotic community in the initial soil but also after the biodegradation test, with 97.8 ± 0.5 % and 91.7 ± 0.4 % ($p < 0.05$), respectively. The remaining 16S rRNA sequences were assigned to *Archaea*, presenting a relative abundance of 1.5 ± 0.1 % and 6.6 ± 0.2 % ($p < 0.05$) in the inoculum soil and after the incubation, respectively. *Proteobacteria* and *Actinobacteriota* are often indicated as the main dominant *Bacteria* in soils (Li et al., 2022), which was observed in this work. Out of 24 bacterial phyla identified, the most abundant phylum was *Actinobacteria* (41.5 ± 6.6 %), followed by *Proteobacteria* (28.0 ± 0.4 %) and *Acidobacteria* (7.6 ± 2.6 %) in the initial soil as presented in Table S1 (Appendix from Chapter 3). After the incubation assays, this order was changed to *Proteobacteria* (31.2 ± 0.5 %) ($p < 0.05$), *Actinobacteria* (19.8 ± 3.2 %), and *Acidobacteria* (10.0 ± 3.4 %). This shows a significant shift in the soil microbial composition induced by the presence of the plastic blend. The relative abundance of *Chloroflexi* (Table S2 - Appendix from Chapter 3) and *Acidobacteria* increased, and these tend to be predominant in oligotrophic environments (Ho et al., 2017). The degradation of PHBV probably caused the release of high quantities of 3-hydroxybutyric acid (one monomer of PHBV) which may slightly reduce the pH that is beneficial to *Acidobacteria.* Most archaeal sequences belong to *Crenarchaeota* representing 1.2 % ± 0.2 % of the prokaryotic community in the initial soil and 4.5 ± 0.6 % ($p < 0.05$) after the assay (Table S2 - Appendix from Chapter 3) All *Bacteria* or *Archaea* phyla which present statistically significant differences in the soil before and after adding the PHBV/PBAT film ($p < 0.05$) are presented in Table S2 (Appendix from Chapter 3). The degradation of the PHBV/PBAT film was associated with an increase in the relative abundance of *Firmicutes*, which have been described to grow well in environments of high carbon availability (Cleveland et al., 2007; Jenkins et al., 2010). On the other hand, the relative abundance of *Bacteroidetes*, which are broadly disseminated in soils and are experts in degrading complex organic matter, decreased in relative abundance (Huang et al., 2019).

Regarding the eukaryotic community, *Fungi* were predominant with 61.8 ± 2.0 % and 26.0 ± 0.8 % ($p < 0.05$) in the initial soil and after the assay respectively. This variation is conditioned by the percentage of sequences classified as “no hit” (i.e., which could not be assigned to any microbe) that increased 40 %. Therefore, probably the differences in the percentages presented are not as significant as they may seem to be. The most abundant phyla within the *Fungi* domain were *Ascomycota* (45.7± 6.1 %) and *Basidiomycota* (12.8 ± 3.8 %) in the initial soil as shown in Table S3 (Appendix from Chapter 3). After the biodegradation assay, the relative abundance decreased significantly ($p < 0.05$) to 16.7 ± 2.2 % for

Ascomycota and 5.9 ± 1.8 % for *Basidiomycota*. In the *Eukaryota*, the *Apicomplexa* phylum was the most represented in both soils, with 1.7 ± 0.3 % for the initial soil and 1.4 ± 0.2 % after the soil assay. After incubation with the plastic blend only the phylum *Blastocladiomycota* (*Fungi*) significantly increased ($p < 0.05$) while *Xanthophyceae* (*Eukaryota*) and *Chytridiomycota* (*Fungi*) decreased ($p < 0.05$).

The microorganisms presenting the highest relative abundance (top 20) and that showed statistical differences in the soil before and after the addition of the PHBV/PBAT film, are presented in Tables 3.6 and 3.7.

Table 3.6 Taxonomical classification of the top 20 microorganism species (*Bacteria* or *Archaea*) that have statistically significant differences in the soil before and after adding the PHBV/PBAT film ($p < 0.05$)

Taxonomic identification (species)	Relative abundance (%)						Ratio
	Inoculum			After Incubation			
Unclassified Chlamydiae	< 0.01	±	< 0.01	0.04	±	0.01	21.79
Synechococcus sp	< 0.01	±	< 0.01	0.03	±	< 0.01	19.60
Fusobacterium sp	< 0.01	±	< 0.01	0.06	±	0.01	18.60
Unclassified Campylobacterales	< 0.01	±	< 0.01	0.08	±	0.02	15.65
Levilinea sp	< 0.01	±	< 0.01	0.06	±	0.02	12.77
Methylosinus sporium	0.02	±	< 0.01	0.21	±	0.03	11.78
Unclassified Gammaproteobacteria	0.08	±	0.02	0.85	±	0.19	11.04
Unclassified Archaea	0.13	±	0.02	1.18	±	0.14	9.11
Melghirimyces thermohalophilus	< 0.01	±	< 0.01	0.04	±	0.01	8.77
Unclassified Cytophagaceae	< 0.01	±	< 0.01	0.01	±	< 0.01	8.36
Nitratireductor sp	0.03	±	0.01	0.24	±	0.10	8.28
Woodsholea maritima	< 0.01	±	< 0.01	0.03	±	0.01	8.01
Dokdonella fugitiva	0.02	±	0.01	0.15	±	0.08	7.77
Ohtaekwangia sp	0.04	±	0.02	0.33	±	0.13	7.37
Thiorhodospira sp	0.12	±	0.04	0.87	±	0.32	7.27
Candidatus Metachlamydia lacustris	< 0.01	±	< 0.01	0.02	±	0.01	6.85
Cohnella laeviribosi	0.01	±	< 0.01	0.04	±	0.02	6.30
Thermobacillus sp	< 0.01	±	< 0.01	0.01	±	< 0.01	6.25
Azoarcus sp	0.01	±	< 0.01	0.09	±	0.01	6.02
Unclassified Alphaproteobacteria	0.09	±	0.02	0.55	±	0.11	5.95

In total 210 prokaryotic species and 81 eukaryotic species, presenting significant differences in their relative abundance before and after soil incubation could be identified These species are not described in the literature as PBAT or PHBV degraders, but the ratio of relative abundance (calculated considering

the relative abundance at the end of the incubation and that in the inoculum soil) increased between 5.9 and 21.8 (prokaryotic) or 2.8 and 29.9 (eukaryotic), Nonetheless, the majority of these microbial species which significantly increase their relative abundance are still present in low relative abundances (< 1 %).

Table 3.7 Taxonomical classification of the top 20 microorganism species (eukaryotes) that have statistically significant differences in the soil before and after adding the PHBV/PBAT film ($p < 0.05$)

Taxonomic identification (species)	Relative abundance (%)						Fold
	Inoculum			After Incubation			
Cavernomonas stercoris	< 0.01	±	< 0.01	0.20	±	0.07	29.91
Hamigera striata	< 0.01	±	< 0.01	0.07	±	0.03	18.05
Aspergillus versicolor	0.07	±	0.04	1.07	±	0.61	15.75
Rigidoporus vinctus	< 0.01	±	< 0.01	0.02	±	0.01	15.35
Penicillium paradoxum	0.01	±	< 0.01	0.13	±	0.03	14.67
Balamuthia mandrillaris	< 0.01	±	< 0.01	0.05	±	0.01	12.40
Platyreta germanica	0.04	±	< 0.01	0.41	±	0.03	9.40
Allas diplophysa	0.01	±	0.01	0.11	±	0.05	7.51
Aspergillus candidus	0.03	±	0.01	0.19	±	0.07	7.12
Metabolomonas insania	0.01	±	< 0.01	0.05	±	0.02	5.97
Onygena equina	< 0.01	±	< 0.01	0.01	±	0.01	5.05
Exophiala sp.	0.52	±	0.20	2.60	±	0.99	4.96
Protostelium arachisporum	0.01	±	< 0.01	0.02	±	0.01	4.59
Ochromonas sp.	< 0.01	±	< 0.01	< 0.01	±	< 0.01	3.74
Spongospora subterranea	0.25	±	0.02	0.85	±	0.08	3.43
Prototheca cutis	0.01	±	< 0.01	0.05	±	0.01	3.21
Nannochloris bacillaris	0.01	±	< 0.01	0.02	±	0.01	3.09
Clitopilus cf scyphoides	< 0.01	±	< 0.01	0.01	±	< 0.01	3.07
Cercomonas edax	0.07	±	0.03	0.21	±	0.08	2.96
Acanthamoeba genotype	0.01	±	< 0.01	0.02	±	0.00	2.82

The species with a significant increase in their relative abundance after the assay include *Cavernomonas stercoris* (< 0.01 ± < 0.01 % to 0.20 ± 0.07 %), *Hamigera striata* (< 0.01 ± < 0.01 % to 0.07 ± 0.03 %), Unclassified *Chlamydiae* (< 0.01 ± < 0.01 % to 0.04 ± 0.01 %) and *Synechococcus* sp. (< 0.01 ± < 0.01 % to 0.03 ± < 0.01 %). These results motivate the investigation in future studies, of what could be the role of these species on the polymer biodegradation.

3.3.4 Analysis of microbial community changes at the genus level

The most abundant genus among *Fungi* whose abundance ratio increased from the inoculum soil to the end of the incubation was *Aspergillus* (1.07 ± 0.34 % to 2.33 ± 0.74 %). Other genera assigned to *Fungi* also increased in abundance and were present in percentages higher than 1 %. These were the following: *Exophiala* (0.52 ± 0.20 % to 2.60 ± 0.99 %) ($p < 0.05$), unknown *Basidiomycota* (0.35 ± 0.40 % to 1.72 ± 1.96 %) and *Talaromyces* (0.64 ± 0.24 % to 1.69 ± 0.64 %) as indicated in Table 3.8.

Table. 3.8 Taxonomic identification of the genera identified by the 18S rRNA gene sequencing with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (genus)	Relative abundance (%)	
	Inoculum	After Incubation
Cladosporium	12.13 ± 1.15	0.04 ± < 0.01
No Hit	10.94 ± 2.76	49.81 ± 12.58
Boeremia	4.77 ± 3.42	0.00 ± 0.00
Alternaria	4.75 ± 1.82	0.07 ± 0.02
Unknown *Filobasidiaceae*	3.90 ± 2.13	0.00 ± 0.00
Unknown *Pyronemataceae*	2.46 ± 0.90	1.77 ± 0.65
Tetracladium	2.27 ± 3.21	0.00 ± 0.00
Phoma	2.19 ± 0.61	0.39 ± 0.11
Unknown *Ascomycota*	1.81 ± 0.17	0.50 ± 0.05
Penicillium	1.69 ± 0.56	1.65 ± 0.55
Unknown *Tremellales*	1.69 ± 0.59	0.88 ± 0.31
Unclassified *Eimeriidae*	1.52 ± 0.21	1.20 ± 0.16
Parastagonospora	1.44 ± 0.58	0.44 ± 0.18
Cryptococcus	1.40 ± 0.71	1.25 ± 0.63
Tricholoma	1.34 ± 0.53	0.31 ± 0.13
Unknown *Pleosporales*	1.33 ± 0.23	0.30 ± 0.05
Didymella	1.29 ± 0.73	0.55 ± 0.31
Ophiosphaerella	1.26 ± 0.31	0.09 ± 0.02
Aspergillus	1.07 ± 0.34	2.33 ± 0.74
Unknown	0.92 ± 0.30	0.91 ± 0.29
Unknown Streptophyta	0.05 ± 0.07	3.24 ± 4.55
Exophiala	0.52 ± 0.20	2.60 ± 0.99
Unknown *Basidiomycota*	0.35 ± 0.40	1.72 ± 1.96
Talaromyces	0.64 ± 0.24	1.69 ± 0.64
Rhizopus	0.88 ± 0.09	1.12 ± 0.27
Others	39.83 ± 0.04	37.53 ± 10.53

The *Bacteria* of the most represented genera (> 1 %) are indicated in Table 3.9.

Table 3.9 Taxonomic identification of the genera identified by the 16S rRNA gene sequencing with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (genus)	Relative abundance (%)	
	Inoculum	After Incubation
Unknown *Bacteria*	8.83 ± 0.83	14.32 ± 1.34
Arthrobacter	8.59 ± 0.79	2.76 ± 0.25
Unknown *Actinobacteria*	5.92 ± 0.63	4.12 ± 0.44
Sphingomonas	3.57 ± 0.53	1.04 ± 0.16
Terrabacter	3.54 ± 0.77	0.66 ± 0.14
Nocardioides	3.24 ± 0.68	0.28 ± 0.06
Unclassified *Acidobacteriia*	2.95 ± 1.20	3.87 ± 1.57
Unknown *Betaproteobacteria*	2.38 ± 0.11	4.61 ± 0.20
Unknown *Actinobacteria*	1.72 ± 0.52	1.21 ± 0.36
Unknown *Acidobacteriia*	1.69 ± 0.56	1.79 ± 0.59
Streptomyces	1.62 ± 0.44	1.71 ± 0.47
Unclassified *Bacteria*	1.55 ± 0.19	2.85 ± 0.36
Massilia	1.55 ± 0.33	0.18 ± 0.04
Bacillus	1.52 ± 0.46	2.20 ± 0.67
Phycicoccus	1.32 ± 0.53	0.19 ± 0.08
Mycobacterium	1.27 ± 0.38	0.88 ± 0.26
Unclassified *Crenarchaeota*	1.19 ± 0.15	4.52 ± 0.59
Unknown *Rhizobiales*	1.04 ± 0.09	2.48 ± 0.22
Marmoricola	1.02 ± 0.11	0.11 ± 0.01
Unknown *Bacteroidetes*	0.98 ± 0.06	0.47 ± 0.03
Unknown *Acidobacteriia*	0.77 ± 0.03	1.88 ± 0.08
Unknown *Bacilli*	0.81 ± 0.03	1.30 ± 0.04
Unknown *Actinobacteria*	0.89 ± 0.36	1.19 ± 0.48
Unknown *Alphaproteobacteria*	0.63 ± 0.08	1.02 ± 0.13
Unclassified *Acidobacteriales*	0.49 ± 0.09	1.02 ± 0.20
Unknown *Bacteroidetes*	0.17 ± 0.04	1.01 ± 0.23
Unclassified *Acidobacteria*	0.10 ± 0.01	1.00 ± 0.09
Bradyrhizobium	0.71 ± 0.17	0.99 ± 0.24
Unclassified *Betaproteobacteria*	0.71 ± 0.16	0.99 ± 0.22
Unknown *Acidobacteriales*	0.66 ± 0.16	0.96 ± 0.24
Others	38.55 ± 2.08	38.40 ± 2.07

The genera of *Bacteria* initially in the soil (Table 3.9). that increased the most in relative abundance were Unclassified *Crenarchaeota*, Unknown *Alphaproteobacteria*, Unknown *Betaproteobacteria*, *Massilia*, *Bacillus* and Unclassified *Acidobacteriia.* The differences were all significant ($p < 0.05$) except for the last two. The genera that increased the most in relative abundance and became most represented (> 1 %) were by this order unknown *Archaea*, unknown *Proteobacteria* and unclassified *Acidobacteriales*.

3.3.5 Isolation and identification of PHBV degraders

In total, 12 colony types showed clearing zones on PHBV-containing agar plates, with 9 *Bacteria* and 3 *Fungi* identified (Table 3.10). Five of the isolates were assigned to the *Variovorax* genus, four to *Streptomyces*, one to *Purpureocillium lilacinum* (formerly known as *Paecilomyces lilacinus*) and two to *Aspergillus*. These last isolates were very closely related to *Aspergillus pseudodeflectus* which were not previously identified as capable of degrading PHBV. Although plates containing PBAT films were used no PBAT degraders could be isolated from this soil. In Figure S1 - Appendix from Chapter 3 it is possible to see the PHBV plates with and without colonies.

Table 3.10 Taxonomic identification of the isolates with the ability to biodegrade PHBV based on the similarity between partial 16S rRNA or ITS sequences obtained by sequencing and those present in the NCBI database

Isolates		**Sequence alignment results**		
Isolate designation	**Sequence Length (bp)**	**Closely related species**	**Genbank ID**	**Identity (%)**
1	1397	*Variovorax* sp. strain DAIF27	MW079396	99.93
		Variovorax sp. strain DAIF22	MW079391	
		Variovorax sp. 3A_12	AY689027	
		Variovorax paradoxus strain E4C	AF209469	
		Variovorax sp. WFF52	AB003627	
5	1405	*Variovorax* sp. strain DAIF22	MW079391	99.43
9	1386	*Variovorax* sp. strain DAIF27	MW079396	99.28
		Variovorax sp. strain DAIF22	MW079391	
		Variovorax sp. 3A_12	AY689027	
		Variovorax paradoxus strain E4C	AF209469	
		Variovorax sp. WFF52	AB003627	
11	1400	*Variovorax paradoxus* strain FW305-C-20-3	MT160407	99.79
14	1398	*Variovorax* sp. strain DAIF27	MW079396	99.79
		Variovorax sp. strain DAIF22	MW079391	
		Variovorax sp. 3A_12	AY689027	
		Variovorax paradoxus strain E4C	AF209469	
		Variovorax sp. WFF52	AB003627	
6	1398	*Streptomyces* sp. E5N408	KX279639	99.43

		Streptomyces sp. E4N372	KX279637	
		Streptomyces viridochromogenes strain NRRL B-24167	EU812168	
		Streptomyces sp. b26	EU260042	
8	1397	*Streptomyces* sp. strain TN71	MG198761	100
		Streptomyces sp. TN82	LT608133	
		Streptomyces pseudogriseolus strain YR-37	KY753277	
		Streptomyces sp. X4-15	KT581289	
12	1397	*Streptomyces* sp. strain TN71	MG198761	100
		Streptomyces sp. TN82	LT608133	
		Streptomyces pseudogriseolus strain YR-37	KY753277	
		Streptomyces sp. X4-15	KT581289	
21	1390	*Streptomyces* sp. strain TN71	MG198761	100
		Streptomyces sp. TN82	LT608133	
		Streptomyces pseudogriseolus strain YR-37	KY753277	
		Streptomyces sp. X4-15	KT581289	
18	606	*Purpureocillium lilacinum* isolate DSM100329_DF58_RLCS20	MT453285	99.83
		Purpureocillium lilacinum strain C. C. Lee AgF3-7	MH793580	
		Purpureocillium lilacinum culture CBS:226.73B strain CBS 226.7	MH860675	
		Purpureocillium lilacinum	OU989487	
		Purpureocillium sp. 2 BRO-2013	KF367485	
		Fungal sp. isolate E496_ITS	OK161079	
		Purpureocillium lilacinum strain M3516	KC157741	
		Purpureocillium lilacinum strain M3297	KC157738	
		Purpureocillium sp. isolate UH.1552.194	MZ374606	
		Purpureocillium sp. isolate UH.1552.175	MZ374592	
		Purpureocillium sp. isolate UH.1552.171	MZ374588	
		Purpureocillium sp. isolate UH.1552	MZ374583	
		Purpureocillium lilacinum strain 139E	MT732891	
		Paecilomyces lilacinus isolate B3A	HM242262	
		Paecilomyces lilacinus	AB103380	
		Purpureocillium lilacinum clone SF_397	MT52967	
22	586	*Aspergillus* sp. 8 BRO-2013	KF367554	100
		Aspergillus pseudodeflectus strain CBS 756.74	OL711759	
		Aspergillus pseudodeflectus isolate CCMG111	MH790295	
		Aspergillus ustus strain UOA/HCPF 9236	FJ878630	
23	571	*Aspergillus pseudodeflectus* strain CMV006F9	MK450644	100
		*Aspergillus calidoustu*s strain CGMCC 3.05313	MN650836	
		Aspergillus calidoustus strain CGMCC 3.05298	MN650835	
		Aspergillus sp. *isolate* CNUFC-RD103	MW480243	
		Aspergillus calidoustus strain CBS 113228	OL711783	
		Aspergillus pseudodeflectus isolate ASF-147	MT957546	

	Aspergillus sp. 085241B	JF312217

3.4 DISCUSSION

This study demonstrates the ability of PHBV to be biodegraded in soil, which motivates its incorporation in packaging materials. The bilayer material presented a faster biodegradation, in the first months (Figure 3.3b) probably due to the PHBV presence in the soil, but as this material disappeared, the rate slowed down, since PBAT biodegradation in the soil is considerably more difficult. The characterization techniques (Table 3.3, 3.4, 3.5) showed that the PHBV was not present on the final residue. The carbonyl absorbance reduction detected after incubation in the ATR-FTIR analysis is typically observed in some soil, compost and other incubations and indicates also that PBAT has been degraded (Kijchavengkul et al., 2010a, 2010b; Qi et al., 2021; Weng et al., 2013b). The formation of free O-H at 3694 cm^{-1} and 3620 cm^{-1}, and free OOH (peroxide) at 3436 cm^{-1} detected (Table 3.3), are normally due to the main chain scission from biodegradation and/or hydrolysis at ester linkages with the production of smaller oligomers, which can easily permeate out of the polymer matrix (Kijchavengkul et al., 2010a). The decrease in T_d (Table 3.4) is probably due to the decreased molecular weight, resulting from the molecular chain fracture after degradation (Fu et al., 2020). Weng et al., (2013b). obtained a similar result with PBAT (incubated in soil), although the differences were lower (1 °C). The increased crystallinity (Table 3.5) found, occurs with other polymers, indicating that the amorphous regions are more susceptible to biodegradation than the crystalline regions (Kijchavengkul et al., 2010b; Palsikowski et al., 2018). Kijchavengkul et al., (2010a) observed after PBAT composting conditions that the aliphatic unit (BA), formed by adipic acid and 1,4 butanediol, was more vulnerable to hydrolysis and biodegradation than the rigid aromatic unit (BT) formed by the dimer, terephthalic acid and 1,4-butanediol. Probably fewer compact structures allow faster biodegradation and hydrolysis of the PBAT amorphous regions. The BA units and BT units share a common crystal lattice by adapting the chain conformation of the BA units into the BT crystal lattice to form a co-crystallization structure (Fu et al., 2020). The biodegradation of PBAT tends to decrease as the quantity of aromatic components increase. That makes sense since the BT unit increases the crystallinity (Witt et al., 1997). The SEM images revealed *Fungi* colonization of the film surface (Figure 3.4). *Fungi* can release extracellular enzymes, that are known to be effective in PHA assimilation (Oda et al., 1995). The studies on PBAT biodegradation in soil also reported the dominance of *Fungi*, suggesting an important role of these microorganisms in PBAT biodegradation in soil (Muroi et al., 2016; Nikolić et al., 2017).

Several taxa were identified as PHBV degraders confirming that PHBV is degraded by a broad spectrum of microorganisms. Concerning the genus *Aspergillus*, *A. fumigatus* is commonly indicated as a PHBV degrader, and *A. ustus* can biodegrade PHAs, but this result was found for PHB (Gonda et al., 2000; Al Hosni et al., 2019). Nonetheless, in this work, it was possible to identify closely related *Aspergillus* species capable of degrading PHBV, that have not been reported before (Table 3.10), namely *Aspergillus pseudodeflectus and*. *Aspergillus* sp*. 8 BRO-2013*. *Streptomyces, Variovorax* and *Purpureocillium*, were also found in this work as PHBV degraders. Microorganisms of the same genera have been identified before as PHBV degraders for example *Variovorax paradoxus* and *Purpureocillium lilacinum,* isolated from several types of soils (Boyandin et al., 2012a, 2013; Charnock, 2021; Mergaert et al., 1993; Sang et al., 2002). *Streptomyces* spp*.* for example are known for the production of a wide range of enzymes and secondary metabolites, being not surprising the ability to degrade polymers (Calabia and Tokiwa, 2004; Shao et al., 2019).

Concerning the community analysis at the genera level (Table 3.8 and 3.9), PBAT and PHBV were found to increase the abundance of microorganisms closely related to *Penicillium*, *Fusarium*, and *Aspergillus* in composting conditions (Zhou et al., 2022). In this work, an increase in the relative abundance of *Aspergillus* (1.07 ± 0.34 % to 2.33 ± 0.74 % fungal community) was detected and their importance in the biodegradation was confirmed by isolating 2 isolates assigned to *Aspergillus* (Table 3.10). The species enriched in soil after the film incubation were *Aspergillus versicolor* ($p < 0.05$)*, Aspergillus candidus* ($p < 0.05$)*, Aspergillus niger* and *Aspergillus cervinus,* but these species are not reported as PHBV or PBAT degrader except for *Aspergillus niger* isolated from garden soil and capable of degrading PHB (Kumaravel et al., 2010). In other studies, other microorganisms of the same genus such as *Aspergillus fumigatus* were identified as PHBV degraders (Mergaert et al., 1993). In the case of *Streptomyces* and *Variovorax*, besides having several isolates found here as capable of degrading PHBV, these genera have also been indicated as PHBV degraders in other works (Mergaert and Swings, 1996; Mergaert et al., 1993; Suyama et al., 1998). Nonetheless, their relative abundance remained the same (1.62 ± 0.44 to 1.71 ± 0.47) or in the case of *Variovorax* even decreased slightly during the biodegradation assays.

Regarding the microbial community, some species already described as PHBV degraders were identified, and their relative abundance increased from the inoculum soil to the end of the biodegradation assay. This include species of the genera *Brevibacillus* (0.04 ± 0.01 to 0.11 ± 0.04) ($p < 0.05$), *Talaromyces* (0.64 ± 0.24 to 1.69 ± 0.64)*, Rhodococcus* (0.21 ± 0.07 to 0.21 ± 0.08), *Ralstonia* (0.01 ± 0.01 to 0.03 ± 0.03) and *Xanthomonas* (< 0.01 ± < 0.01 to < 0.01 ± < 0.01), all indicated as PHA degraders

(Boyandin et al., 2012b; Nishide et al., 1999; Suyama et al., 1998). *Brevibacillus thermoruber* increased 4.5 times ($p < 0.05$), *Brevibacillus borstelensis* almost doubled, and *Brevibacillus sediminis* increased to 1.6 times, *Rhodococcus rhodochrous* and *Rhodococcus equi* more than doubled their abundance, *Talaromyces funiculosus, and Talaromyces purpureogenus* ($p < 0.05$) were also enriched, and only *Rhodococcus* sp. was reduced. *Nocardiopsis* sp. increased more than 9.5 times, and its genera have been identified as capable of degrading PHB and PHBV in soils (Boyandin et al., 2013). Several bacteria from the genus *Bacillus* native to sewage and soil have been reported as capable of degrading PHBV and even some polymerases have been purified (Boyandin et al., 2012a; Shah et al., 2007). Normally the species indicated in these cases are mostly strains from *Bacillus* sp. but for example, *Bacillus cereus* has also been identified. This genus was enriched from 1.52 to 2.2 times including all the identified species, *Bacillus graminis, Bacillus licheniformis,* and *Bacillus* sp. (the most represented) except *Bacillus foraminis.* This result may indicate that *Bacillus* has an important role in the biodegradation of the PHBV layer. Several species of the genera *Streptomyces, Cupriavidus, Burkholderia* and *Penicillium* are described as capable of degrading PHB or PHBV in soils, such as *Penicillum* sp., *Cupriavidus* sp., *Burkholderia* sp., *Penicillium simplicissimum, Penicillium oxalicum, Penicillium funiculosum,* and *Penicillium lilacinus* (Boyandin et al., 2013; Brucato and Wong, 1991; Mergaert et al., 1993; Satti et al., 2020). However, their relative abundance remained almost the same. *Penicillium paradoxum* increased 14 times ($p < 0.05$), although the level remained very low (0.03). The genera *Gongronella* had a slight increase, and species from this genera, e.g. *Gongronella butleri*, have already been indicated as PHA degraders in tropical soils (Boyandin et al., 2013). Some of the genera identified as PHAs degraders in soils, namely *Mycobacterium*, *Stenotrophomonas*, *Acinetobacter*, *Fusarium*, *Cryptococcus, Pseudomonas*, *Duganella*, *Cladosporium, Agrobacterium*, and *Terrabacter* were present in the soil analysed (Boyandin et al., 2012b; Jendrossek et al., 1996; Mergaert and Swings, 1996; Suyama et al., 1998; Tareq, 2010). Nonetheless, these genera were not enriched by the film's presence in the soil and their relative abundance even decreased. Probably they did not have the enzymes capable of degrading PHAs or this PHBV in particular, since some microorganisms can only degrade a type of PHA (Volova et al., 2017), or they are not cultivable. The genera *Arthrobacter* presented in Table 3.9, had a marked reduction superior to 3 times in relative abundance, although it has been indicated as a PHA degrader (Asano and Watanabe, 2014)

It was not possible to isolate PBAT degraders but some genera and species with microorganisms capable of degrading PBAT were identified. Several strains of *Bacillus and* actinomycetes have been identified from soil or compost as capable of degrading PBAT (Kanwal et al., 2022; Witt et al., 2001). The relative

abundance of *Bacillus* increased during the biodegradation assays. The existing scientific knowledge indicates the strong possibility that PBAT degradation in soil is the result of the synergistic activity of different organisms. This was verified, for example, by Šerá et al. (2020), with *Thermobispora bispora* (thermophilic actinomycetes) and *Bacilli species.* When these species were tested individually, they could not carry out the biodegradation. This could justify why in this work no isolates for PBAT were obtained since the isolation deprives the microorganisms of their necessary interactions and synergetic activity to accomplish the biodegradation.

In other study, two main bacterial genera of PBAT-degraders were identified after 7 months of incubated PBAT, *Azospirillum* and *Mesorhizobium* (Muroi et al., 2016). In this work, *Mesorhizobium* increased in relative abundance, although maintaining low levels, 0.151 ± 0.048 % to 0.234 ± 0.075 %. Han et al. (2021) indicated that *Bradyrhizobium*, *Ramlibacter*, and *Variovorax* genera are potential degraders of PBAT in soils since they are possible sources of PBAT hydrolase genes and were enriched in a surface of PBAT film buried in the soil. *Bradyrhizobium* was significantly enriched with PBAT buried in farmland soil (Zhang et al., 2022), and it also increased from 0.7 ± 0.2 to 1.0 ± 0.2 % here. The genera *Rhizopus* increased slightly in relative abundance, namely *Rhizopus oryzae,* but *Rhizopus microspores* tripled. A strain of *Rhizopus oryzae* has an enzyme capable of degrading PBAT (Zumstein et al., 2017), and it may also be acting here as a potential degrader.

Fungi of the genera *Trichoderma*, *Acremonium*, *Verticillium*, and *Zygosporium*, commonly indicated in the literature as capable of degrading PHAS in soils, were not detected by the community analysis (Boyandin et al., 2012b). In contrast, several genera or species of microorganisms were enriched such as *Ralstonia* sp. and *Xanthomonas* sp., including some identified as PBAT and PHBV degraders, nevertheless, it is possible that some may act as commensal organisms, using monomers and/or other degradation products, having thus an essential activity. The results revealed a change in the microbial community due to the addition of PHBV/PBAT films, which may impact the diversity and function of the community. Ong and Sudesh (2016) demonstrated that the diversity of the soil microbial community was linked to the degradation of PHAs (i.e. PHBV). Vannini et al., (2021) indicated that the microbial community on a PHBV surface was different and displayed a lower richness compared with the community from the surrounding sediments.

3.5 CONCLUSION

The PHBV layer conferred the biodegradation character to the PHBV/PBAT film, which was corroborated by all the physicochemical analyses. The PBAT layer, remained more resistant to biodegradation, although with clear signs of biodegradation. Twelve microorganisms capable of degrading PHBV were isolates including, two closely related to Aspergillus species that have not been described before with this capability. However, it was not possible to isolate microorganisms involved in the PBAT biodegradation. The development of new protocols may help to identify, PBAT-degrading microorganisms. The soil community, changed by the addition of the polymers, and in future works, it is important to understand if this shift is advantageous to the soil in terms of fertility since the diversity and type of soil microbial community can be correlated with the degradation of polymers. The results confirm the PHAs biodegradation potential and may be important for the selection of these green plastics as packaging solutions especially when considering the advantages when entering the environment.

3.6 REFERENCES

Al Hosni, A. S., Pittman, J. K., & Robson, G. D. (2019). Microbial degradation of four biodegradable polymers in soil and compost demonstrating polycaprolactone as an ideal compostable plastic. *Waste Management*, 97, 105–114. Retrieved from https://doi.org/10.1016/j.wasman.2019.07.042

Arcos-Hernandez, M. V, Laycock, B., Pratt, S., Donose, B. C., Nikolić, M. A. L., Luckman, P., … Lant, P. A. (2012). Biodegradation in a soil environment of activated sludge derived polyhydroxyalkanoate (PHBV). *Polymer Degradation and Stability*, 97(11), 2301–2312. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.07.035

Asano, Y., & Watanabe, S. (2014). Isolation of Poly (3-Hydroxybutyrate) (PHB) - degrading Microorganisms and Characterization of PHB-depolymerase from Arthrobacter sp . strain. *Bioscience, Biotechnology, and Biochemistry ISSN:*, 8451(May), 1191–1194. Retrieved from https://doi.org/10.1271/bbb.65.1191

ASTM D1293-18. (2018). Standard Test Methods for pH of Water. *ASTM International*

ASTM D425-17. (2017). Test Method for Centrifuge Moisture Equivalent of Soils. *ASTM International*

ASTM D5988-18. (2018). Standard Test Method for Determining Aerobic Biodegradation in Soil of Plastic Materials. *ASTM International*

Beber, V. C., de Barros, S., Banea, M. D., Brede, M., de Carvalho, L. H., Hoffmann, R., … Wellen, R. M. R. (2018). Effect of Babassu natural filler on PBAT/PHB biodegradable blends: An investigation of thermal, mechanical, and morphological behavior. *Materials*, 11(5). Retrieved from https://doi.org/10.3390/ma11050820

Boyandin, A N, Prudnikova, S. V, Filipenko, M. L., Khrapov, E. A., Vasil'ev, A. D., & Volova, T. G. (2012a). Biodegradation of polyhydroxyalkanoates by soil microbial communities of different structures and detection of PHA degrading microorganisms. *Applied Biochemistry and Microbiology*, 48(1), 28–36. Retrieved from https://doi.org/10.1134/S0003683812010024

Boyandin, Anatoly N, Prudnikova, S. V, Karpov, V. A., Ivonin, V. N., Đỗ, N. L., Nguyễn, T. H., … Gitelson, I. I. (2013). Microbial degradation of polyhydroxyalkanoates in tropical soils. *International*

Biodeterioration & Biodegradation, 83, 77–84. Retrieved from https://doi.org/https://doi.org/10.1016/j.ibiod.2013.04.014

Boyandin, Anatoly N, Rudnev, V. P., Ivonin, V. N., Prudnikova, S. V, Korobikhina, K. I., Filipenko, M. L., … Sinskey, A. J. (2012b). Biodegradation of Polyhydroxyalkanoate Films in Natural Environments. *Macromolecular Symposia*, 320(1), 38–42. Retrieved from https://doi.org/10.1002/masy.201251004

Brucato, C. L., & Wong, S. S. (1991). Extracellular poly(3-hydroxybutyrate) depolymerase from Penicillium funiculosum: General characteristics and active site studies. *Archives of Biochemistry and Biophysics*, 290(2), 497–502. Retrieved from https://doi.org/https://doi.org/10.1016/0003-9861(91)90572-Z

Calabia, B. P., & Tokiwa, Y. (2004). Microbial degradation of poly(D-3-hydroxybutyrate) by a new thermophilic streptomyces isolate. *Biotechnology Letters*, 26(1), 15–19. Retrieved from https://doi.org/10.1023/b:bile.0000009453.81444.51

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, 108, 4516–4522. Retrieved from https://doi.org/10.1073/pnas.1000080107

Charnock, C. (2021). Norwegian soils and waters contain mesophilic, plastic-degrading bacteria. *Microorganisms*, 9(1), 1–18. Retrieved from https://doi.org/10.3390/microorganisms9010094

Chivrac, F., Kadlecová, Z., Pollet, E., & Avérous, L. (2006). Aromatic copolyester-based nano-biocomposites: Elaboration, structural characterization and properties. *Journal of Polymers and the Environment*, 14(4), 393–401. Retrieved from https://doi.org/10.1007/s10924-006-0033-4

Cleveland, C. C., Nemergut, D. R., Schmidt, S. K., & Townsend, A. R. (2007). Increases in soil respiration following labile carbon additions linked to rapid shifts in soil microbial community composition. *Biogeochemistry*, 82(3), 229–240. Retrieved from https://doi.org/10.1007/s10533-006-9065-z

Cunha, M., Fernandes, B., Covas, J. A., Vicente, A. A., & Hilliou, L. (2016). Film blowing of PHBV blends and PHBV-based multilayers for the production of biodegradable packages. *Journal of Applied Polymer Science*, 133(2). Retrieved from https://doi.org/10.1002/app.42165

Ferreira-Santos, P., Ibarz, R., Fernandes, J.-M., Pinheiro, A. C., Botelho, C., Rocha, C. M. R., … Martín-Belloso, O. (2021). Encapsulated Pine Bark Polyphenolic Extract during Gastrointestinal Digestion: Bioaccessibility, Bioactivity and Oxidative Stress Prevention. *Foods*, 10(2), 1–19. Retrieved from https://doi.org/10.3390/foods10020328

Fu, Y., Wu, G., Bian, X., Zeng, J., & Weng, Y. (2020). Biodegradation Behavior of Poly(Butylene Adipate-Co-Terephthalate) (PBAT), Poly(Lactic Acid) (PLA), and Their Blend in Freshwater with Sediment. *Molecules*, 25(17). Retrieved from https://doi.org/10.3390/molecules25173946

Fukushima, K., Wu, M. H., Bocchini, S., Rasyida, A., & Yang, M. C. (2012). PBAT based nanocomposites for medical and industrial applications. *Materials Science and Engineering C*, 32(6), 1331–1351. Retrieved from https://doi.org/10.1016/j.msec.2012.04.005

Gonçalves, S. P. C., Martins-Franchetti, S. M., & Chinaglia, D. L. (2009). Biodegradation of the Films of PP, PHBV and Its Blend in Soil. *Journal of Polymers and the Environment*, 17(4), 280. Retrieved from https://doi.org/10.1007/s10924-009-0150-y

Gonda, K. E., Jendrossek, D., & Molitoris, H. P. (2000). Fungal degradation of the thermoplastic polymer poly-β-hydroxybutyric acid (PHB) under simulated deep sea pressure. *Hydrobiologia*, 426(1), 173–183. Retrieved from https://doi.org/10.1023/A:1003971925285

Hall, T. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98. Retrieved from

https://doi.org/10.1021/bk-1999-0734.ch008

Han, Y., Teng, Y., Wang, X., Ren, W., Wang, X., Luo, Y., ... Christie, P. (2021). Soil Type Driven Change in Microbial Community Affects Poly(butylene adipate- co-terephthalate) Degradation Potential. *Environmental Science and Technology*, 55(8), 4648–4657. Retrieved from https://doi.org/10.1021/acs.est.0c04850

Ho, A., Di Lonardo, D. P., & Bodelier, P. L. E. (2017). Revisiting life strategy concepts in environmental microbial ecology. *FEMS Microbiology Ecology*, 93(3), fix006. Retrieved from https://doi.org/10.1093/femsec/fix006

Huang, Y., Zhao, Y., Wang, J., Zhang, M., Jia, W., & Qin, X. (2019). LDPE microplastic films alter microbial community composition and enzymatic activities in soil. *Environmental Pollution*, 254, 112983. Retrieved from https://doi.org/https://doi.org/10.1016/j.envpol.2019.112983

ISO 18400. (2018). Soil quality. *International Organization for Standardization*

Javadi, A., Srithep, Y., Lee, J., Pilla, S., Clemons, C., Gong, S., & Turng, L. S. (2010). Processing and characterization of solid and microcellular PHBV/PBAT blend and its RWF/nanoclay composites. *Composites Part A: Applied Science and Manufacturing*, 41(8), 982–990. Retrieved from https://doi.org/10.1016/j.compositesa.2010.04.002

Jendrossek, D., Schirmer, A., & Schlegel, H. G. (1996). Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology*, 46(5), 451–463. Retrieved from https://doi.org/10.1007/s002530050844

Jenkins, S. N., Rushton, S. P., Lanyon, C. V., Whiteley, A. S., Waite, I. S., Brookes, P. C., ... O'Donnell, A. G. (2010). Taxon-specific responses of soil bacteria to the addition of low level C inputs. *Soil Biology and Biochemistry*, 42(9), 1624–1631. Retrieved from https://doi.org/10.1016/j.soilbio.2010.06.002

kanwal, A., Zhang, M., Sharaf, F., & Chengtao, L. (2022). Screening and characterization of novel lipase producing Bacillus species from agricultural soil with high hydrolytic activity against PBAT poly (butylene adipate co terephthalate) co-polyesters. *Polymer Bulletin*, (0123456789). Retrieved from https://doi.org/10.1007/s00289-021-03992-4

Kasuya, K. ichi, Ishii, N., Inoue, Y., Yazawa, K., Tagaya, T., Yotsumoto, T., ... Nagai, D. (2009). Characterization of a mesophilic aliphatic-aromatic copolyester-degrading fungus. *Polymer Degradation and Stability*, 94(8), 1190–1196. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.04.013

Kijchavengkul, T., Auras, R., Rubino, M., Alvarado, E., Camacho Montero, J. R., & Rosales, J. M. (2010a). Atmospheric and soil degradation of aliphatic-aromatic polyester films. *Polymer Degradation and Stability*, 95(2), 99–107. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.11.048

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2010b). Biodegradation and hydrolysis rate of aliphatic aromatic polyester. *Polymer Degradation and Stability*, 95(12), 2641–2647. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.07.018

Kumaravel, S., Hema, R., & Lakshmi, R. (2010). Production of polyhydroxybutyrate (Bioplastic) and its biodegradation by pseudomonas lemoignei and aspergillus niger. *E-Journal of Chemistry*, 7(S1), 1–4. Retrieved from https://doi.org/10.1155/2010/148547

Lammi, S., Gastaldi, E., Gaubiac, F., & Angellier-Coussy, H. (2019). How olive pomace can be valorized as fillers to tune the biodegradation of PHBV based composites. *Polymer Degradation and Stability*, 166, 325–333. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2019.06.010

Lane, D. J. (1991). Nucleic Acid Techniques in Bacterial Systematic. In E. Stackebrandt, E. and Goodfellow, M. (Ed.), *Nucleic Acid Techniques in Bacterial Systematic* (pp. 115–175). New York: John Wiley and Sons Inc.

Larsson, M., Markbo, O., & Jannasch, P. (2016). Melt processability and thermomechanical properties of blends based on polyhydroxyalkanoates and poly(butylene adipate-: Co -terephthalate). *RSC Advances*, 6(50), 44354–44363. Retrieved from https://doi.org/10.1039/c6ra06282b

Li, C., Cui, Q., Li, Y., Zhang, K., Lu, X., & Zhang, Y. (2022). Effect of LDPE and biodegradable PBAT primary microplastics on bacterial community after four months of soil incubation. *Journal of Hazardous Materials*, 429(January), 128353. Retrieved from https://doi.org/10.1016/j.jhazmat.2022.128353

Li, G., Shankar, S., Rhim, J. W., & Oh, B. Y. (2015). Effects of preparation method on properties of poly(butylene adipate-co-terephthalate) films. *Food Science and Biotechnology*, 24(5), 1679–1685. Retrieved from https://doi.org/10.1007/s10068-015-0218-5

Malik, K. A. (1988). A new freeze-drying method for the preservation of nitrogen-fixing and other fragile bacteria. Journal of Microbiological Methods, 8(5), 259–271. Retrieved from https://doi.org/https://doi.org/10.1016/0167-7012(88)90008-5

Medlin, L., Elwood, H. J., Stickel, S., & Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71(2), 491–499. Retrieved from https://doi.org/https://doi.org/10.1016/0378-1119(88)90066-2

Mergaert, J., & Swings, J. (1996). Biodiversity of microorganisms that degrade bacterial and synthetic polyesters. *Journal of Industrial Microbiology*, 17(5), 463–469. Retrieved from https://doi.org/10.1007/BF01574777

Mergaert, J., Webb, A., Anderson, C., Wouters, A., & Swings, J. (1993). Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. *Applied and Environmental Microbiology*, 59(10), 3233–3238. Retrieved from https://doi.org/10.1128/aem.59.10.3233-3238.1993

Muroi, F., Tachibana, Y., Kobayashi, Y., Sakurai, T., & Kasuya, K. I. (2016). Influences of poly(butylene adipate-co-terephthalate) on soil microbiota and plant growth. *Polymer Degradation and Stability*, 129, 338–346. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2016.05.018

Nagarajan, V., Misra, M., & Mohanty, A. K. (2013). New engineered biocomposites from poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)/poly(butylene adipate-co-terephthalate) (PBAT) blends and switchgrass: Fabrication and performance evaluation. *Industrial Crops and Products*, 42(1), 461–468. Retrieved from https://doi.org/10.1016/j.indcrop.2012.05.042

Nar, M., Staufenberg, G., Yang, B., Robertson, L., Patel, R. H., Varanasi, V. G., & D'Souza, N. A. (2014). Osteoconductive bio-based meshes based on Poly(hydroxybutyrate-co- hydroxyvalerate) and poly(butylene adipate-co-terephthalate) blends. *Materials Science and Engineering C*, 38(1), 315–324. Retrieved from https://doi.org/10.1016/j.msec.2014.01.047

Nikolić, M. A. L., Gauthier, E., Colwell, J. M., Halley, P., Bottle, S. E., Laycock, B., & Truss, R. (2017). The challenges in lifetime prediction of oxodegradable polyolefin and biodegradable polymer films. *Polymer Degradation and Stability*, 145, 102–119. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2017.07.018

Nishide, H., Toyota, K., & Kimura, M. (1999). Effects of soil temperature and anaerobiosis on degradation of biodegradable plastics in soil and their degrading microorganisms. *Soil Science and Plant Nutrition*, 45(4), 963–972. Retrieved from https://doi.org/10.1080/00380768.1999.10414346

Oda, Y., Asari, H., Urakami, T., & Tonomura, K. (1995). Microbial degradation of poly(3-hydroxybutyrate) and polycaprolactone by filamentous fungi. *Journal of Fermentation and Bioengineering*, 80(3),

265–269. Retrieved from https://doi.org/10.1016/0922-338X(95)90827-M

Ong, S. Y., & Sudesh, K. (2016). Effects of polyhydroxyalkanoate degradation on soil microbial community. *Polymer Degradation and Stability*, 131, 9–19. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2016.06.024

Pal, A. K., Wu, F., Misra, M., & Mohanty, A. K. (2020). Reactive extrusion of sustainable PHBV/PBAT-based nanocomposite films with organically modified nanoclay for packaging applications: Compression moulding vs. cast film extrusion. *Composites Part B: Engineering*, 198(May), 108141. Retrieved from https://doi.org/10.1016/j.compositesb.2020.108141

Palsikowski, P. A., Kuchnier, C. N., Pinheiro, I. F., & Morales, A. R. (2018). Biodegradation in Soil of PLA/PBAT Blends Compatibilized with Chain Extender. *Journal of Polymers and the Environment*, 26(1), 330–341. Retrieved from https://doi.org/10.1007/s10924-017-0951-3

Qi, R., Jones, D. L., Liu, Q., Liu, Q., Li, Z., & Yan, C. (2021). Field test on the biodegradation of poly(butylene adipate-co-terephthalate) based mulch films in soil. *Polymer Testing*, 93, 107009. Retrieved from https://doi.org/10.1016/j.polymertesting.2020.107009

Reddy, S. V., Thirumala, M., & Mahmood, S. K. (2009). A novel Bacillus sp. accumulating poly (3-hydroxybutyrate-co-3- hydroxyvalerate) from a single carbon substrate. *Journal of Industrial Microbiology and Biotechnology*, 36(6), 837–843. Retrieved from https://doi.org/10.1007/s10295-009-0561-8

Rosa, D. S., Lotto, N. T., Lopes, D. R., & Guedes, C. G. F. (2004). The use of roughness for evaluating the biodegradation of poly-β-(hydroxybutyrate) and poly-β-(hydroxybutyrate-co-β-valerate). *Polymer Testing*, 23(1), 3–8. Retrieved from https://doi.org/10.1016/S0142-9418(03)00042-4

Rose, R. S., Richardson, K. H., Latvanen, E. J., Hanson, C. A., Resmini, M., & Sanders, I. A. (2020). Microbial degradation of plastic in aqueous solutions demonstrated by Co2 evolution and quantification. *International Journal of Molecular Sciences*, 21(4). Retrieved from https://doi.org/10.3390/ijms21041176

Russo, P., Vetrano, B., Acierno, D., & Mauro, M. (2013). Thermal and structural characterization of biodegradable blends filled with halloysite nanotubes. *Polymer Composites*, 34(9), 1460–1470. Retrieved from https://doi.org/10.1002/pc.22419

Salvador, A. F., Cavaleiro, A. J., Paulo, A. M. S., Silva, S. A., Guedes, A. P., Pereira, M. A., … Alves, M. M. (2019). Inhibition Studies with 2-Bromoethanesulfonate Reveal a Novel Syntrophic Relationship in Anaerobic Oleate Degradation. *Applied and Environmental Microbiology*, 85(2), e01733-18. Retrieved from https://doi.org/10.1128/AEM.01733-18

Sang, B. I., Hori, K., Tanji, Y., & Unno, H. (2002). Fungal contribution to in situ biodegradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) film in soil. *Applied Microbiology and Biotechnology*, 58(2), 241–247. Retrieved from https://doi.org/10.1007/s00253-001-0884-5

Santos, C., Santos da Silva, B. N., Amorim Ferreira e Ferreira, A. F., Santos, C., Lima, N., & Silva Bentes, J. L. (2020). Fungal Endophytic Community Associated with Guarana (Paullinia cupana Var. Sorbilis): Diversity Driver by Genotypes in the Centre of Origin. *Journal of Fungi*, 6(3). Retrieved from https://doi.org/10.3390/jof6030123

Satti, S. M., Shah, Z., Luqman, A., Hasan, F., Osman, M., & Shah, A. A. (2020). Biodegradation of Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Newly Isolated Penicillium oxalicum SS2 in Soil Microcosms and Partial Characterization of Extracellular Depolymerase. *Current Microbiology*, 77(8), 1622–1636. Retrieved from https://doi.org/10.1007/s00284-020-01968-7

Šerá, J., Kadlečková, M., Fayyazbakhsh, A., Kučabová, V., & Koutný, M. (2020). Occurrence and analysis of thermophilic poly(Butylene adipate-co-terephthalate)-degrading microorganisms in temperate

zone soils. *International Journal of Molecular Sciences*, 21(21), 1–17. Retrieved from https://doi.org/10.3390/ijms21217857

Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2007). Isolation and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) degrading bacteria and purification of PHBV depolymerase from newly isolated Bacillus sp. AF3. *International Biodeterioration and Biodegradation*, 60(2), 109–115. Retrieved from https://doi.org/10.1016/j.ibiod.2007.01.004

Shao, H., Chen, M., Fei, X., Zhang, R., Zhong, Y., Ni, W., ... Tan, X. (2019). Complete Genome Sequence and Characterization of a Polyethylene Biodegradation Strain, Streptomyces Albogriseolus LBX-2. *Microorganisms*, 7(10), 1–13. Retrieved from https://doi.org/10.3390/microorganisms7100379

Silva, Pedro M, Martins, A. J., Fasolin, L. H., & Vicente, A. A. (2021). Modulation and Characterization of Wax-Based Olive Oil Organogels in View of Their Application in the Food Industry. *Gels*, 7(1), 1–18. Retrieved from https://doi.org/10.3390/gels7010012

Silva, P M, Prieto, C., Andrade, C. C. P., Lagarón, J. M., Pastrana, L. M., Coimbra, M. A., ... Cerqueira, M. A. (2022). Hydroxypropyl methylcellulose-based micro- and nanostructures for encapsulation of melanoidins: Effect of electrohydrodynamic processing variables on morphological and physicochemical properties. *International Journal of Biological Macromolecules*, 202, 453–467. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.01.019

Sin, M. C., Gan, S. N., Annuar, M. S. M., & Tan, I. K. P. (2010). Thermodegradation of medium-chain-length poly(3-hydroxyalkanoates) produced by Pseudomonas putida from oleic acid. *Polymer Degradation and Stability*, 95(12), 2334–2342. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.08.027

SM 2540 G. (1998). Total, Fixed, and Volatile Solids in Solid and Semisolid Samples. *Standard Methods for the Examination of Water and Wastewater, APHA-AWWA-WPCF. (20th ed.)*

Suyama, T., Tokiwa, Y., Ouichanpagdee, P., Kanagawa, T., & Kamagata, Y. (1998). Phylogenetic Affiliation of Soil Bacteria That Degrade Aliphatic Polyesters Available Commercially as Biodegradable Plastics. *Applied and Environmental Microbiology*, 64(12), 5008–5011. Retrieved from https://doi.org/10.1128/AEM.64.12.5008-5011.1998

Tabasi, R. Y., & Ajji, A. (2015). Selective degradation of biodegradable blends in simulated laboratory composting. *Polymer Degradation and Stability*, 120(February), 435–442. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.07.020

Tareq, A. Z. (2010). Comparative study on biodegradation of some synthetic polymers blended with some different naturally occurring polymers. *Thesis*

Thomas, S., Shumilova, A. A., Kiselev, E. G., Baranovsky, S. V., Vasiliev, A. D., Nemtsev, I. V., ... Volova, T. G. (2019). Thermal, mechanical and biodegradation studies of biofiller based poly-3-hydroxybutyrate biocomposites. *International Journal of Biological Macromolecules*, 155(15), 1373–1384. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.11.112

Urbanek, A. K., Rymowicz, W., Strzelecki, M. C., Kociuba, W., Franczak, Ł., & Mirończuk, A. M. (2017). Isolation and characterization of Arctic microorganisms decomposing bioplastics. *AMB Express*, 7(1), 148. Retrieved from https://doi.org/10.1186/s13568-017-0448-4

Vannini, C., Rossi, A., Vallerini, F., Menicagli, V., Seggiani, M., Cinelli, P., ... Balestri, E. (2021). Microbial communities of polyhydroxyalkanoate (PHA)-based biodegradable composites plastisphere and of surrounding environmental matrix: a comparison between marine (seabed) and coastal sediments (dune sand) over a long-time scale. *Science of the Total Environment*, 764, 142814. Retrieved from https://doi.org/10.1016/j.scitotenv.2020.142814

Volova, T. G., Prudnikova, S. V., Vinogradova, O. N., Syrvacheva, D. A., & Shishatskaya, E. I. (2017). Microbial Degradation of Polyhydroxyalkanoates with Different Chemical Compositions and Their

Biodegradability. *Microbial Ecology*, 73(2), 353–367. Retrieved from https://doi.org/10.1007/s00248-016-0852-3

Wallace, P. W., Haernvall, K., Ribitsch, D., Zitzenbacher, S., Schittmayer, M., Steinkellner, G., … Birner-Gruenberger, R. (2017). PpEst is a novel PBAT degrading polyesterase identified by proteomic screening of Pseudomonas pseudoalcaligenes. *Applied Microbiology and Biotechnology*, 101(6), 2291–2303. Retrieved from https://doi.org/10.1007/s00253-016-7992-8

Weng, Y.-X., Wang, L., Zhang, M., Wang, X.-L., & Wang, Y.-Z. (2013a). Biodegradation behavior of P(3HB,4HB)/PLA blends in real soil environments. *Polymer Testing*, 32(1), 60–70. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymertesting.2012.09.014

Weng, Y. X., Jin, Y. J., Meng, Q. Y., Wang, L., Zhang, M., & Wang, Y. Z. (2013b). Biodegradation behavior of poly(butylene adipate-co-terephthalate) (PBAT), poly(lactic acid) (PLA), and their blend under soil conditions. *Polymer Testing*, 32(5), 918–926. Retrieved from https://doi.org/10.1016/j.polymertesting.2013.05.001

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. In *PCR Protocols* (Vol. 31, pp. 315–322). Elsevier. Retrieved from https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Witt, Uwe, Müller, R. J., & Deckwer, W. D. (1997). Biodegradation behavior and material properties of aliphatic/aromatic polyesters of commercial importance. *Journal of Environmental Polymer Degradation*, 5(2), 81–89. Retrieved from https://doi.org/10.1007/BF02763591

Witt, U., Einig, T., Yamamoto, M., Kleeberg, I., Deckwer, W. D., & Müller, R. J. (2001). Biodegradation of aliphatic-aromatic copolyesters: Evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. *Chemosphere*, 44(2), 289–299. Retrieved from https://doi.org/10.1016/S0045-6535(00)00162-4

Zhang, Y., Ma, J., O'Connor, P., & Zhu, Y.-G. (2022). Microbial communities on biodegradable plastics under different fertilization practices in farmland soil microcosms. *Science of The Total Environment*, 809, 152184. Retrieved from https://doi.org/10.1016/j.scitotenv.2021.152184

Zhou, Y., Sun, Y., Liu, J., Ren, X., Zhang, Z., & Wang, Q. (2022). Effects of microplastics on humification and fungal community during cow manure composting. *Science of the Total Environment*, 803, 150029. Retrieved from https://doi.org/10.1016/j.scitotenv.2021.150029

Zumstein, M. T., Rechsteiner, D., Roduner, N., Perz, V., Ribitsch, D., Guebitz, G. M., … Sander, M. (2017). Enzymatic Hydrolysis of Polyester Thin Films at the Nanoscale: Effects of Polyester Structure and Enzyme Active-Site Accessibility. *Environmental Science & Technology*, 51(13), 7476–7485. Retrieved from https://doi.org/10.1021/acs.est.7b01330

CHAPTER 4.

BIODEGRADATION OF PHB/PBAT FILMS AND ISOLATION OF NOVEL PBAT BIODEGRADERS FROM SOIL MICROBIOMES

4.1 INTRODUCTION

Plastics are used in several applications, particularly in the food packaging market. Blends and composites of biodegradable polymers have been developed to replace conventional plastics that have a significant footprint, whether because they are produced from fossil fuels or because they are not biodegradable (Pietrini et al., 2007). PHB has a high production cost and brittleness, which are some of the main factors that hinder its extensive application (Tokiwa and Calabia, 2007). To overcome this, PHB is often blended with other polyesters, to reduce the cost of production and improve characteristics such as toughness, while maintaining the biodegradation behaviour (Liu et al., 2019; Tokiwa and Calabia, 2007). Despite the numerous advantages of PBAT, it is more difficult to biodegrade, and it is considered a compostable plastic (Witt et al., 2001), as indicated in section 2.4.4. Indeed, only a few species isolated from the soil are described thus far as PBAT degraders, for example, *Bacillus subtilis*, *Rhodococcus* sp. strain NKCM 2511, *Bacillus pumilus* and *Stenotrophomonas* sp. YCJ1 (Jia et al., 2021; Muroi et al., 2017; Soulenthone et al., 2020; Trinh Tan et al., 2008). PBAT biodegradability is favored under thermophilic conditions, which is not verified in the majority of natural environments where plastics are disposed (Šerá et al., 2020a).

The PHB/polybutylene adipate terephthalate (PBAT) blend offers numerous advantages when applied to food packaging. For instance, PHB offers similar properties to conventional plastics, including stiffness, high degree of crystallinity, and melting point (Tokiwa and Calabia, 2007), while PBAT presents thermal stability, among other relevant properties (Beber et al., 2018). As an example, PBAT/PHB blends with and without Babassu filler (palm tree), demonstrated similar maximum deformation at break at lower temperatures, which are extremely interesting properties for packing and food containers, since they may have to preserve suitable elongations at low temperatures (Beber et al., 2018). The biodegradability of PHB/PBAT blends was tested before under composting conditions by Tabasi and Ajji, (2015) and a value of 50 % was achieved in only one month. The approach of using other polymers together with PBAT was proved as a good alternative, to accelerate the process of biodegradation. Šerá et al., (2016) showed that PBAT filled with 25 % starch obtained 53 % degradation, with more than 20 % exceeding the starch content. Wu (2012) described an increase from 20 % weight loss for PBAT to more than 80 % in 120 days for the material with PBAT and cellulose acetate. The same material, but with maleic anhydride-grafted, although with superior mechanical properties, presented almost 10 % less weight loss. Nevertheless, in soil environments, where plastic waste is frequently discarded, these blends or

composites were never tested. In this work, the biodegradation of a PHB/PBAT bilayer film by soil microbiomes was evaluated, and microorganisms degrading PHB and PBAT were successfully isolated.

4.2 MATERIALS AND METHODS

4.2.1 Soil collection and characterization

The soil was sampled at the University of Minho (Campus of Gualtar, Braga, Portugal) in October of 2020, with typical temperatures and conditions for the season (ranging from 10 °C to 22 °C). All the procedures were done exactly as described in section 3.2.1 Soil media of Chapter 3. The only difference was that the soil was sieved to 1 mm. The physicochemical characteristics are presented in the Table S1 (Appendix from Chapter 4).

4.2.2 Biodegradation experiments set-up

All the procedures were done exactly as described in section 3.2.2 90 Biodegradation experiments set-up of Chapter 3, except for the inoculated soil in the bioreactors that was 200 mg. The bioreactor was set up according to Figure 4.1.

Figure 4.1 Representation of a bioreactor.

The plastic used was a PHB/PBAT 55/45 % in weight bilayer in the form of film (11.3 × 9 cm with 35 μm of thickness). The PHB was an experimental PHB grade (Biomer P309) supplied by Biomer (Krailling, Germany). The PBAT was a commercial aliphatic-aromatic copolyester-based polymer (EcoflexVR F blend C1200, a film-blowing grade). This film was developed for food packaging, the PHB was produced from food industry by-products, and the details about the co-extrusion of the bilayer film can be found in the

work from Teixeira et al. (2020). Different amounts of the blend and cellulose paper (9 × 8.75 cm with 197 μm of thickness) were added to the bioreactors to obtain an equivalent mass of organic carbon, according to Table 4.1.

Table 4.1 Weight and total carbon content of the materials tested. All experiments were performed in triplicate

Bioreactor	Test Material	Carbon (%)	Total Amount of test material (mg)	Total carbon of test material (mg)
PHB/PBAT Film	PHB (45 %) PBAT (55 %)	60.9[1]	500.3 ± 1.2	304.6 ± 0.2
Cellulose filter paper	Cellulose (100 %)	44.0[2]	685.1 ± 0.6	304.5 ± 0.3

1 - Determined by elemental analysis. 2 - Determined considering its chemical composition ($C_6H_{10}O_5$)

4.2.3 Analytical and microscopic methods

All the methods were applied as described in section 3.2.3 Analytical and microscopic methods of Chapter 3.

4.2.4 Agar plate containing plastic

For the preparation of the PHB plates, the methodology was the same as the PHBV plates, described in section 3.2.4 Agar plate containing plastic of Chapter 3. Concerning the PBAT plates, 2 g of PBAT pellets (Ecoflex) were dissolved in 40 mL of chloroform ≥99.8 % analytical reagent grade (Fisher Scientific). This solution was poured into 100 mL of distilled water containing 20 mg of N-Lauroylsarcosine sodium salt, 95 % (Acros Organics), and blended in an Ultra-Turrax (14.000 RPM, 5 min), and then put in an ultrasound bath (10 min). The N-Lauroylsarcosine sodium salt has been already used for the same purpose with other polymers (Charnock, 2021). About 70 mL of molten agar was added to the previous solution and then incubated overnight at 30 °C under continuous stirring to eliminate all the organic solvent. Finally, this solution was added to solution B (described before) and autoclaved. The solutions A, C, and D were autoclaved separately and added, creating a similar medium to the PHB medium.

4.2.5 Isolation of microorganisms degrading the polymer film

All the methods were applied as described in section 3.2.5 Isolation of microorganisms degrading the polymer film of Chapter 3.

4.2.6 DNA isolation, polymerase chain reaction (PCR) and Sanger and Illumina sequencing

All the methods were applied as described in section 3.2.6 DNA isolation, polymerase chain reaction (PCR) and Sanger and Illumina sequencing of Chapter 3. All FASTA files obtained from Sanger sequencing (corresponding to the microbial isolates) and FASTQ files obtained from Illumina sequencing (corresponding to the taxonomic analysis of soil microbial communities) have been submitted to ENA under the accession numbers PRJEB62456 and PRJEB60405, respectively. The accession numbers of the ITS and 16S rRNA sequences obtained via Sanger sequencing for the PHB isolates were ERZ21821875 and ERZ21821876, respectively. For the PBAT degraders, the accession was ERZ21821877.

4.2.7 Statistical analysis

All the statistics were applied as described in section 3.2.7 Statistical analysis of Chapter 3.

4.3 RESULTS

4.3.1 Biodegradation assay

The requirements of the ASTM D5988-18 (2018) for the physicochemical properties of the soil, namely the pH (6-8) were respected, as indicated in Table 4.2.

Table. 4.2 Physicochemical properties of the soil. All experiments were performed in triplicate

Soil Parameters	
Total dry solids[1] (%)	92.45
Volatile solids[1](%)	7.55
pH[2]	6.53
Total organic carbon amount[3] (%)	2.71
Total nitrogen amount[3] (%)	0.15
Carbon/nitrogen ratio[3] (C:N)	18.07
Moisture holding capacity[4] (%)	19.43

*1 –Determined using the SM 2540 G (1998), *2 – Determined using the ASTM D1293 (2018), *3 –Determined by elemental analysis, *4 – Determined using the ASTM D425 (2017)

After 6 months of incubation, the bilayer film reached an average mineralization of 47 ± 1 % and the reference material 75 ± 1 % as can be seen in Figure 4.2.a. During the first 11 days, the biodegradation

of the PHB/PBAT film was less than 2 % and after that period the rate increased till day 52 and then decreased until the end of the assay. Cellulose was degraded during the first 73 days in circa 64 %. Therefore, the assay is considered valid according to the ASTM D5988 -18 (2018), since more than 70 % of the reference material (in this case cellulose) was biodegraded in less than 180 days (it took approximately 150 days). during the last 50 days of the assay, the biodegradation increased only by 5 %. These results show that the inoculum soil could biodegrade polymeric compounds and that the microbiome was active and with good hydrolytic capacity.

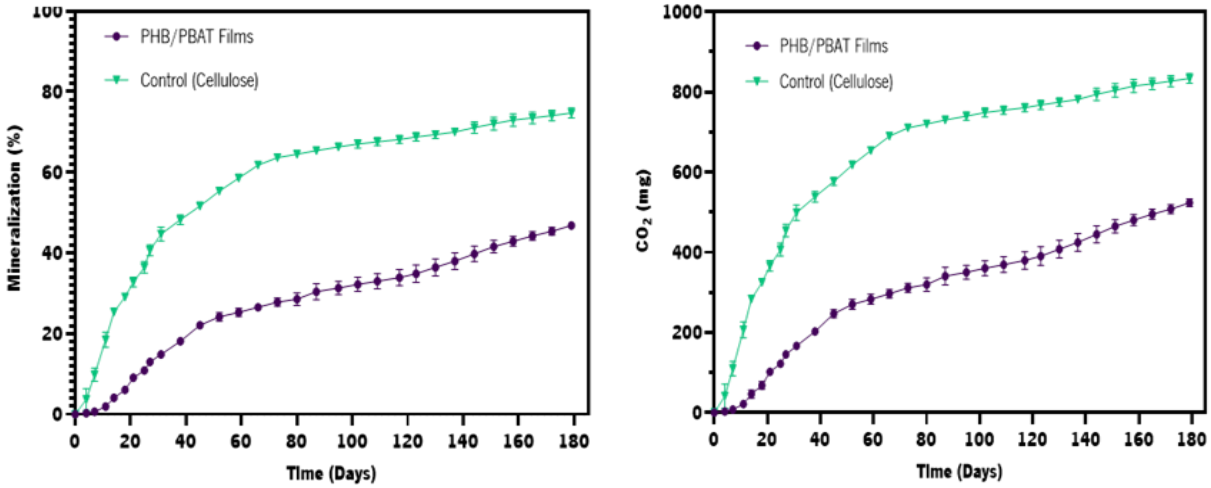

Figure 4.2 a) Carbon mineralization curves (%) of the tested materials, PHB/PBAT films and cellulose (control material) by soil microbiomes. b) Average carbon dioxide (CO_2) evolution of the tested materials. All experiments were performed in triplicate.

The CO_2 evolution of the films and controls throughout the assay presented in Figure 4.2 b revealed that more organic carbon was transformed in CO_2 in the control assay.

4.3.2 Analytical and microscopic results

The films after the incubation period showed several cracks and surface erosion (Figure 4.3d) and some hyphae filaments were also noticed (Figure 4.3c) which are signs of biodegradation. The FTIR analysis only identified spectra corresponding to PBAT, indicating that PHB was totally degraded or converted to smaller polymer sizes which cannot be detected by FTIR.

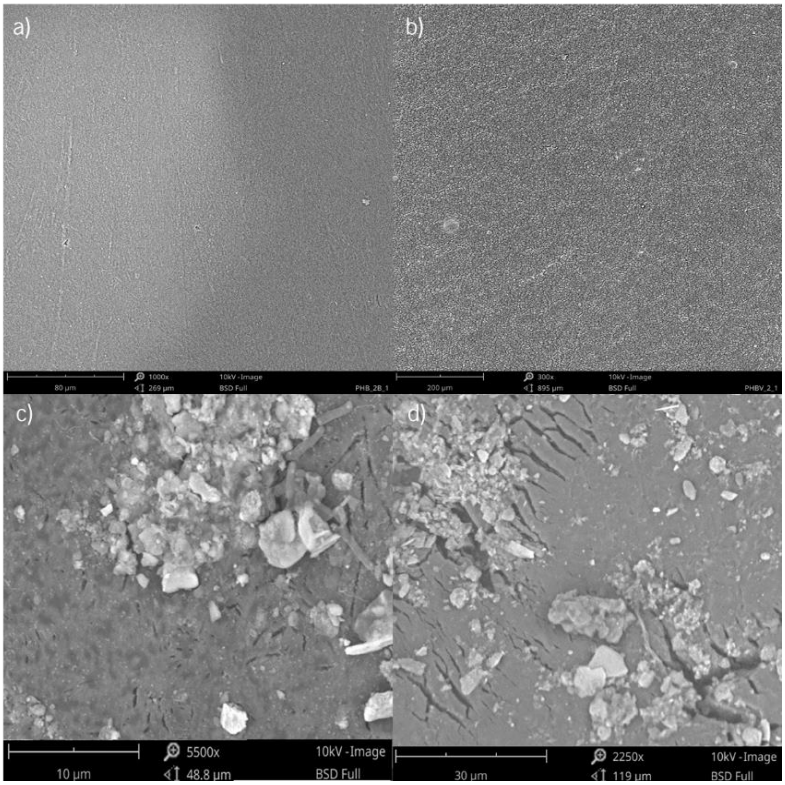

Figure 4.3 Scanning electron micrographs of plastic films after 6 months of incubation in soil. a) PBAT layer before incubation, b) PHB layer before incubation, c) PHB layer after incubation, evidencing the presence of hyphae, and d) PBAT layer after incubation, showing some cracks.

The PBAT IR spectrum after incubation presented small peaks at 3691 cm^{-1} and 3410 cm^{-1}, which are attributed to the formation of free O-H and free OOH (peroxide) respectively. The development of these peaks is normally related to the scission in the main chain resulting from the hydrolysis or the action of enzymes, where oligomers can leave the polymer (Sabapathy et al., 2019). Initially, the PHB layer, presented the characteristic spectrum, as shown in Table 4.3 with the 2974 cm^{-1} (C-H), 1718 cm^{-1} (C=O), and 1273 cm^{-1} (C–O) peaks represented, among others (Mousavioun et al., 2012; Wang et al., 2015). The same happened with PBAT, for example, 2957 cm^{-1} (C-H stretching), 1709 cm^{-1} (stretching vibration of C=O), 1017 cm^{-1} (C-H in-plane deformation vibration), and 726 cm^{-1} (bending vibration of C-H off-plane of the benzene ring) (Mohanty and Nayak, 2010; Qi et al., 2021). However, when comparing the PBAT peaks, it is possible to observe slight reductions in several peaks, such as in the C-H in-plane deformation vibration (1390 cm^{-1}) and the aromatic C–O (1267 cm^{-1}) but no significant differences in the positioning. The carbonyl absorbance peak (1709 cm^{-1}) was also reduced (Kijchavengkul et al., 2010; Souza et al., 2019).

Table 4.3 Infrared spectral analysis of the PHB/PBAT films before and after soil incubation

Wavenumber (cm^{-1})			
PHB side		**PBAT side**	
Before Incubation	**After Incubation**	**Before Incubation**	**After Incubation**
2974			3691
2932			3410
1718		2957	2957
1453		1709	2873
1379		1505	1709
1358		1457	1578
1273		1409	1505
1260		1390	1457
1225		1267	1409
1178		1250	1390
1129		1165	1321
1099		1118	1267
1054		1102	1250
1043		1017	1165
977		935	1118
953		917	1102
937		873	1017
910		796	935
894		750	916
839		726	873
825		586	796
726		498	750
678			726
623			632
514			584
459			533
429			498
			470

The thermal stability of the bilayer films decreased over time, and several physical differences happened in the film structure, particularly in the PHB layer, due to the biodegradation in the soil. The TGA indicated that the film before the soil test presented two weight loss steps, between 265.20 – 315.02 °C and 362.38 – 444.50 °C. However, after 6 months, only 1 step was detected (351.83 – 454.33 °C). Usually, 100 % of PBAT samples present decomposition temperature (Td) around 400 °C while for PHB T_d was below 300 °C (Larsson et al., 2016; Masood et al., 2018; Qi et al., 2021); in the present work, the same

was found as shown in Table 4.4. These two steps have also been observed in blends of PHB with PBAT (Larsson et al., 2016). The weight loss of the bilayer film was approximately 32 % (corresponding to PHB) and 47 % (corresponding to PBAT) for the two stages before the soil test. After incubation, the weight loss was 73 % corresponding to PBAT, and showing PHB complete disappearance. At the onset of the PBAT stage, the temperature at which the weight loss starts to be significant slightly increased from 380.83 °C to 383.52 °C. The Td, determined at the maximum weight loss rate of the samples, increased from 409.78 °C to 416.77 °C.

Table 4.4 Thermal properties of the samples before and after degradation, determined by TGA. All experiments were performed in triplicate

Samples	T_{d^1} (°C)	Speed max^1	T_d onset^1 (°C)	weight loss^1 (%)	T_{d2} (°c)	Speed max^2	T_d onset^2 (°C)	weight loss^2 (%)
Initial PHB/PBAT Film	294.9 ± 0.8	17.1 ± 2.6	282.1 ± 1.7	32.44 ± 5.62	409.8 ± 0.7	12.2 ± 0.1	380.8 ± 5.0	46.63 ± 0.1
Final PHB/PBAT Film					416.8 ± 2.9	19.9 ± 1.1	383.5 ± 6.6	73.18 ± 4.24

After the incubations, the PBAT fraction presented some differences regarding DSC analysis, and the PHB layer was not detected in all samples. The heating phase of the samples before the soil test presented 2 peaks, with melting temperatures of 118.1 °C and 172.59 °C, as shown in Table 4.5. These results corresponded to PBAT and PHB, respectively, since PHB typically presents higher values for the parameters evaluated, such as melting temperature or crystallinity (Mousavioun et al., 2012; Weng et al., 2013). Blends of PHB and PBAT also present this behaviour, corresponding to the individual characteristics of each polymer (Beber et al. 2018).

Table 4.5 The thermal properties of the samples before and after incubation were determined by DSC. All experiments were performed in triplicate

Samples	T_{m^1}(°C)	Δh^1 (J/g)	T_m Onset^1 (°C)	T_{c^1} (°C)	X_{c^1} (%)	T_{m^2}(°C)	Δh^2 (J/g)	T_m Onset^2 (°C)	T_{c^2} (°C)	X_{c^2} (%)
Initial PHB/PBAT Film	172.59 ± 0.21	18.4 ± 4.3	168.2 ± 0.4	116.9 ± 0.1	12.6 ± 2.9	118.1 ± 0.2	4.5 ± 0.8	98.2 ± 2.8	67.9 ± 0.4	3.9 ± 0.7
Final PHB/PBAT Film						123.8 ± 1.4	12.5 ± 1.4	100.8 ± 1.6	73.7 ± 1.3	10.9 ± 1.2

The crystallinity decreased since PHB has a superior crystallinity and disappeared after the test. The crystallinity of the PBAT layer of the film increased, which indicates that the amorphous regions were consumed faster because they are more accessible to the enzymes (Kijchavengkul et al., 2010). This phenomenon has been described before, since PBAT is a copolymer with an aliphatic unit (most of the amorphous areas), and an aromatic unit (most of the crystalline areas), that is more resistant to degradation (Wang et al., 2015). The increase in the melting temperature suggests that the biodegradation and hydrolysis of the aliphatic unit occurred, which led to an improved crystalline structure with fewer defects (Fu et al., 2020). Nonetheless, the T_d tends to decrease due to molecular chain fracture occurring during degradation (according to Kanwal et al., 2022), but this was not observed in the present work (Table 4.4).

Considering the increased crystallinity demonstrated by the DSC analysis (Table 4.5) and the appearance and reduction of some peaks revealed in the ATR-FTIR analysis (Table 4.3) it was clear that the PBAT was also degraded.

4.3.3 Microbial diversity of soil before and after the biodegradation experiments

The prokaryotic community was very diverse and the relative abundance of 147 species (belonging to 35 different genera) changed after the incubations, but from those, only 28 species increased their relative abundance. Table S1 (Appendix from Chapter 4) presents the microorganisms whose relative abundance changed most before and after the biodegradation assessment.

The relative abundance of 66 species (20 assigned to *Eukaryota* and 40 to *Fungi*) and 43 genera (18 *Fungi* and 20 *Eukaryota*) presented significant differences ($p < 0.05$) before and after the incubations. The genera that increased significantly were *Aphanoascus* and Claroideoglomus (*Fungi*), *Prototheca* (*Plantae*), *Allas,* and Unclassified *Heterolobosea* (Other *Eukaryota*), and all the others decreased. However, the relative abundance of 37 species increased significantly ($p < 0.05$). Table 4.6 shows the list of the species with the biggest differences between relative abundances (Ratio), with *Antrodia albida* increasing more than 120 times.

Table 4.6 Taxonomical identification of the soil eukaryotic microorganisms with the biggest differences in terms of relative abundance

Taxonomic identification (Species)	Relative abundance (%)		
	Inoculum	After Incubation	Ratio*
Antrodia albida	0.01 ± 0.01	0.89 ± 0.01	123.82
Dacrymyces stillatus	0.01 ± 0.01	0.58 ± 0.01	105.28
Waitea circinata	0.02 ± 0.02	0.69 ± 0.02	42.33
Mycoemilia scoparia	0.11 ± 0.04	1.99 ± 0.11	18.51
Talaromyces radicus	< 0.01 ± < 0.01	0.04 ± < 0.01	8.20
Rosulabryum capillare	0.06 ± 0.01	0.42 ± 0.02	7.40
Taxonomic identification (Genus)	**Inoculum**	**After Incubation**	**Ratio**
Cladosporium	0.58 ± 0.05	0.02 ± 0.02	0.03

*Ratio – relative abundance after incubation / relative abundance in the inoculum

The 5 most abundant genera of eukaryotes detected in the inoculum decreased after incubation (Table 4.7). Only Unclassified *Eimeriidae* (6.15 %) and *Aspergillus* (3.54 %) remained, and *Melastiza* (1.31 %), *Ascobolus* (0.84 %), and Unclassified *Eukaryota* (1.23 %) left the top 5. The genera *Oribatula*, *Mycoemilia*, and *Penicillium* (2.64 %) increased and were among the five most represented genera.

Table 4.7 Taxonomical identification of the genera identified by the 18S or 16S rRNA gene sequencing with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (18S rRNA gene sequencing)	Relative abundance (%)	
	Inoculum	After Incubation
No Hit	25.28 ± 1.60	35.53 ± 5.31
Penicillium	1.81 ± 0.26	2.64 ± 0.78
Trinema	1.03 ± 0.10	1.07 ± 0.46
Unclassified *Eimeriidae*	6.15 ± 0.31	2.98 ± 0.49
Aspergillus	3.54 ± 0.43	3.36 ± 1.04
Melastiza	3.06 ± 0.87	1.31 ± 0.73
Ascobolus	2.01 ± 0.88	0.84 ± 0.13
Unclassified *Eukaryota*	1.96 ± 0.20	1.23 ± 0.27
Mortierella	1.94 ± 0.34	0.81 ± 0.24
Talaromyces	1.78 ± 0.50	1.43 ± 0.39
Cryptococcus	1.10 ± 0.22	0.88 ± 0.17
Cercomonas	1.04 ± 0.12	0.67 ± 0.18
Trichosporon	1.01 ± 0.30	0.46 ± 0.20

Alternaria	0.97	±	0.21	0.04	±	0.01
Oribatula	0.61	±	0.79	2.49	±	3.52
Sancassania	< 0.01	±	< 0.01	2.05	±	1.53
Mycoemilia	0.11	±	0.04	1.99	±	1.43
Unclassified *Cercomonadidae*	0.17	±	0.07	1.91	±	2.32
Sistotrema	< 0.01	±	< 0.01	1.12	±	0.80
Arachniotus	0.84	±	0.29	1.10	±	0.02
Peregrinia	0.02	±	0.02	1.03	±	1.43
Others	45.58	±	1.36	35.05	±	0.93

Taxonomic identification (16S rRNA gene sequencing)	**Inoculum**			**After Incubation**		
Unclassified *Bacteria*	7.54	±	0.15	9.74	±	1.1
Unclassified *Crenarchaeota*	2.42	±	0.20	4.50	±	2.1
Bacillus	1.86	±	0.21	2.52	±	1.3
Streptomyces	1.86	±	0.14	3.01	±	1.4
Unclassified *Actinobacteria*	1.78	±	0.16	1.92	±	0.7
No Hit	1.40	±	0.15	1.98	±	0.3
Unclassified *Planctomycetales*	1.39	±	0.10	1.51	±	0.5
Unclassified *Chloroflexi*	1.27	±	0.13	1.42	±	0.1
Unclassified *Rubrobacterales*	1.26	±	0.08	2.10	±	0.7
Unclassified *Rhizobiales*	1.14	±	0.06	1.24	±	0.2
Unclassified *Acidobacteriales*	4.40	±	0.20	2.83	±	0.9
Unclassified *Acidobacteriia*	3.20	±	0.02	2.40	±	1
Sphingomonas	2.40	±	0.12	1.44	±	0.2
Arthrobacter	1.95	±	0.31	1.00	±	0.2
Pseudomonas	1.88	±	0.14	0.16	±	0
Rhodoplanes	1.86	±	0.23	1.53	±	0.1
Unclassified *Actinobacteria*	1.82	±	0.05	1.70	±	0.5
Acidobacterium	1.53	±	0.12	1.09	±	0.3
Unclassified *Deltaproteobacteria*	1.53	±	0.07	1.18	±	0.3
Desulfitobacterium	1.47	±	0.13	0.79	±	0.1
Burkholderia	1.42	±	0.05	1.36	±	0.2
Unclassified *Verrucomicrobia*	1.27	±	0.15	0.83	±	0
Pelobacter	1.25	±	0.03	0.96	±	0.5
Bradyrhizobium	1.17	±	0.08	0.77	±	0.1
Mycobacterium	1.01	±	0.08	0.84	±	0.3
Unclassified *Xanthomonadales*	0.92	±	0.02	1.56	±	0.7
Unclassified *Burkholderiales*	0.87	±	0.05	1.23	±	0.3
Gemmatimonas	0.70	±	0.02	1.18	±	0.2
Unclassified *Archaea*	0.18	±	0.02	1.17	±	0.9
Paenibacillus	0.69	±	0.22	0.93	±	0.6

Others	46.55 ± 0.87	45.12 ± 1.1

After the incubation, the Unclassified *Bacteria* (9.74 %), Unclassified *Crenarchaeota* (4.50 %), and Unclassified *Acidobacteriales* (2.83 %) remained as the most abundant *Bacteria* (Table 4.7), although the first two decreased. On the other hand, Unclassified *Acidobacteriia* and *Sphingomonas* left the five most represented genera being replaced by *Bacillus* (2.52 %) and *Streptomyces* (3.01 %), which increased.

The community is very diverse, with more than 659 and 1007 different microorganisms belonging to different taxonomic groups identified by the 18S rRNA and 16S rRNA sequencing, respectively. The 16S rRNA sequences indicated some variation of *Bacteria* (initial 95.7 ± 0.3 % to 91.8 ± 1.8 % ($p < 0.05$)) and (2.9 ± 0.1 % to 6.2 ± 0.8 % ($p < 0.05$)). In the inoculum soil, the three most abundant phyla were *Proteobacteria* (32.6 ± 0.5 %), *Actinobacteria* (21.2 ± 1.4 %), and *Acidobacteria* (10.7 ± 0.3 %), and this tendency remained after the soil incubation Table S2 - Appendix from Chapter 4). The only phyla that significantly increased ($p < 0.05$) were *Chloroflexi* (from 2.7 % to 3.5 %), Unclassified *Bacteria* (from 7.6 % to 9.8 %), *Gemmatimonadetes* (from 1 % to 1.4 %) and *Fusobacteria* (from 0.02 % to 0.04 %). In contrast, *Tenericutes*, *Deinococcus-Thermus*, *Thermotogae*, *Proteobacteria*, *Armatimonadetes* and *Bacteroidetes* significantly decreased (0.03, 0.007, 0.04, 32.6, 0.02 and 3.9 to 0.01, 0.0004, 0.01, 27.3, 0.004 and 2.2 respectively). Most 16S rRNA gene sequences assigned to *Archaea* belong to the phylum *Crenarchaeot*a and increased the relative abundance from 2.4 ± 0.2 % to 4.5 ± 2.1 %. The relative abundance inside the eukaryotic community at higher taxonomic levels was not significantly affected since the *Fungi* (36.6 %) and *Eukaryota* (24.9 %) decreased but not significantly after the incubation, to 30.6 % and 20.9 % respectively (Table S3 - Appendix from Chapter 4). The most abundant Fungi were the *Basidiomycota* and *Ascomycota*. The only phyla that changed significantly ($p < 0.05$) in terms of relative abundance were *Apicomplexa* (*Eukaryota*), *Xanthophyceae* (*Fungi*) and *Nematoda*, although all decreased. More relevant changes were obtained when analyzing the microbial community at lower taxonomic levels. For instance, the relative abundance of *Antrodia albida, Dacrymyces stillatus, Waitea circinate, Mycoemilia scoparia, Talaromyces radicus and Rosulabryum capillare* increased between 7 and 123 (ratio) after the incubation (Table 4.6).

4.3.4 Isolation and identification of PHB and PBAT degraders

Several microorganisms were isolated with the ability to biodegrade PHB and PBAT. Seven microorganisms, 4 *Fungi* and 3 *Bacteria* were able to form clear zones in agar medium containing PHB (Table 4.8). According to the 16S rRNA gene sequencing, two isolates were assigned to *Streptomyces*, and were closely related to *Streptomyces coelicoflavus* strain SA3120 (99.7 % identity) and *Streptomyces*

sp. strain ACZ2-27 (99.1 % identity), and the other bacterial isolate was assigned to *Variovorax*, more specifically to *Variovorax* strain 369 (97.8 % identity) (Table 4.8).

Table 4.8 Taxonomic identification of the isolates with the ability to biodegrade PHB based on similarity between obtained partial 16S rRNA or ITS sequences and those present in the NCBI database

Isolates		**Sequence alignment results**		
Isolate designation	**Sequence Length (bp)**	**Closely related species**	**Genbank ID**	**Identity (%)**
2	992	*Streptomyces coelicoflavus strain SA3120*	MT355864	99.71
		Streptomyces coelicoflavus strain X6	MT355850	
		Streptomyces graminearus strain NM1	MT071574	
		Streptomyces sp. strain G280	MG917692	
		Actinomyces sp. strain WJM-3	MF170639	
		Streptomyces coelicoflavus strain YB104	MG818968	
		Streptomyces sp. Sn-23	KJ742904	
		Actinomycetia bacterium strain HBU208135	MZ021472	
		Actinomycetia bacterium strain HBU208154	MZ021403	
		Streptomyces sp. GS13	JX465725	
		Streptomyces sp. PW444	HQ684742	
		Streptomyces sp. HaHD12	GQ357942	
11	1064	*Streptomyces sp. ACZ2-27*	LC500236	99.06
		Streptomyces sp. strain GDMCC 60254	MK646063	
		Streptomyces sp. strain JJGP9-13	MK530258	
		Streptomyces sp. strain HRM603-3	MG836203	
6	409	*Purpureocillium lilacinum isolate ET_5*	OP788028	99.14
8	540	*Aspergillus insuetus strain CBS 107.25*	OL711790	99.48
		Aspergillus germanicus strain DTO 179-B4	MN650837	
10	583	*Fungal* sp *strain inoculum M2-1*	MN096589	99.48
		Clonostachys sp. strain daef27	MH550497	
		Clonostachys rosea culture CBS:127294 strain CBS 127294	MH864507	
		Clonostachys rosea culture CBS:126933 strain CBS 126933	MH864340	
15	556	*Fusarium solani isolate N-49-1*	MT560378	99.47
		Fusarium solani clone SF_968	MT530244	
		Fusarium solani clone SF_777	MT530053	
		Fusarium solani clone SF_665	MT529941	
		Fusarium solani clone SF_656	MT529932	
7	1092*	Variovorax sp. strain 369	MG820626	97.82

* - Indicates that only the forward sequence was used in the pairwise alignment

The sequencing of the ITS region allowed to identify 4 *Fungi* closely related to *Fusarium solani* isolate N-49-1 (99.5 % identity), *Purpureocillium lilacinum* isolate ET_5 (99.1 % identity), *Aspergillus insuetus* strain CBS 107.25 (99.5 % identity) and *Clonostachys*. sp. strain daef27 (99.5 % identity). In Figure S1 - Appendix from Chapter 4 is possible to see the PHB plates with and without colonies.

Two different fungi with the ability to biodegrade PBAT could be isolated and were closely related to *Purpureocillium lilacinum* and *Aspergillus pseudodeflectus* as their sequences show very high identify percentages to different species of *Purpureocillium* and *Aspergillus* as is presented in Table 4.9.

Table 4.9 Identification of the PBAT degraders isolates based on similarity searches of partial ITS DNA gene sequences

Isolates		**Sequence alignment results**		
Isolate designation	**Sequence Length (bp)**	**Closely related species**	**Genbank ID**	**Identity (%)**
7	100	*Aspergillus pseudodeflectus isolate CCMG111*	MH790295	100
		Aspergillus sp. 8 BRO-2013	KF367554	
		Aspergillus pseudodeflectus strain CBS 756.74	OL711759	
		Aspergillus ustus strain UOA/HCPF 9236	FJ878630	
9	99.83	*Purpureocillium lilacinum clone SF_357*	MT529633	99.83
		Purpureocillium lilacinum clone SF_327	MT529603	
		Purpureocillium lilacinum strain ZMGRS3	MT446187	
		Purpureocillium lilacinum culture CBS:126685 strain CBS 126685	MH864210	
		Purpureocillium lilacinum isolate M	MG857645	
		Purpureocillium lilacinum isolate 001JFC	KR025540	
		Purpureocillium sp. 1 BRO-2013	KF367471	
		Purpureocillium lilacinum strain M1447	KC157713	
		Purpureocillium sp. isolate UH.1552.164	MZ374582	
		Paecilomyces lilacinus strain CID 004	HQ829056	
		Paecilomyces lilacinus isolate SY45B-a	HM242264	
		Paecilomyces lilacinus strain LTBF 007-1	GQ229080	

4.4 DISCUSSION

This is the first study evaluating the potential of a soil microbiome to biodegrade a blend composed of PBAT and PHB at mesophilic temperatures. PBAT is an interesting polymer that presents great potential, due to its intrinsic characteristics, to be used for food packaging, but has the disadvantage of being hardly

degraded under normal environmental conditions. It is indeed biodegraded under compostable conditions but only slightly biodegraded in soil. For instance, PBAT biodegradation percentages in the soil during a period of 120 days varied from 0.3 to 16 % (Han et al., 2021), which clearly reflects the difficulty of biodegrading PBAT in the soil where plastic waste is commonly discarded. Remarkably, in this work circa 47 % of the PBAT/PHB blend was biodegraded over a period of 180 days (Figure 4.2b). The results indicate that the PHB fraction was more biodegraded (Tables 4.3,4.4 and 4.5) followed by PBAT. The PHB biodegradation was confirmed by the ATR-FTIR, DSC and TGA analyses, which showed that PHB polymer was no longer detected after the assays in the film. This indicates that the PHB layer was cleaved into oligomers and dimers and metabolized by microorganisms, which is not surprising since PHB is the most common polymer of the PHAs family (Carofiglio et al., 2017), and many organisms capable of using it as substrate, including microorganisms assigned to *Fungi* and *Bacteria* (Mergaert and Swings, 1996). The degradation of PHB/PBAT blends in composting revealed the selectivity degradation of the PHB phase (Tabasi and Ajji, 2015). The same type of preference happened in this work. The results indicate that the PBAT, although more resistant, also contributed to the CO_2 evolution. Indeed, SEM images (Figure 4.3) reveal the damage caused to PBAT during the incubations in soil, since no PHB was detected in the final residues, and thus the damaged material corresponds only to PBAT. The FITR and DSC analysis (Tables 4.3 and 4.5) confirmed this result with the development of small peaks related to the scission in the main chain, and the PBAT increased crystallinity related to the degradation of the amorphous regions.

Motivated by these promising results, attempts were made to target the isolation of PBAT-degrading microorganisms from soil. To date, few species have been isolated from soil that can biodegrade PBAT (Jia et al., 2021; Muroi et al., 2017; Soulenthone et al., 2020; Trinh Tan et al., 2008). Furthermore, biodegradation rates reported are usually low: Šerá et al. (2016) indicated 6 % of biodegradation after 100 days of incubation in soil evaluated by CO_2 production. Rychter et al. (2010) indicated about 15 % weight loss after 10 months in standardized sandy soil at 30 °C, and after 22 months the biodegradation increased to 50 %. In the end, two microorganisms were isolated with a proven ability to biodegrade PBAT. The isolates were assigned to the genera *Purpureocillium* and *Aspergillus* (Table 4.9).

This last species, which is assigned to *Aspergillus pseudodeflectus,* was never reported before as a PBAT degrader nor in soil or other environments. In relation to *Purpureocillium lilacinum,* only one strain was indicated as PBAT degrader (*Paecilomyces lilacinus* ATCC 200182). However, the microorganism isolated in this work seems to be a different strain. This result is a step forward in the knowledge of plastic-degrading microbes in natural environments. This is very interesting due to the rarity of discovering and

isolating PBAT-degrading microorganisms. Apart from their contribution to biodegrading PBAT in the soil where they naturally exist, they can be used to enhance the biodegradation of plastic blends containing PBAT. Although PBAT biodegradation under mesophilic conditions is typically a slow process, the appearance of clear zones in agar plates containing PBAT as the sole carbon and energy source, took only 7 days (at 28 °C). From an applied viewpoint, this result is of utmost importance since it shows that if the best growth conditions are provided for these isolates, PBAT can be possibly efficiently biodegraded.

Not many microorganisms significantly change their relative abundance between the beginning and end of the PHB/PBAT biodegradation in soil. Some genera such as *Rhodococcus*, *Clostridium*, *Cryptococcus*, Rhizopus *Stenotrophomonas*, and *Pelosinus* that have species capable of degrading PBAT were also detected in this soil (Aarthy et al., 2018; Biundo et al., 2016; Jia et al., 2021; Muroi et al., 2017; Perz et al., 2016; Zumstein et al., 2017). In this work, from these genera, only *Rhodococcus* and *Clostridium* increased in relative abundance while all the others decreased. It has been found that a consortium with organisms of the genera *Bacillus* (30 % to over 90 %) and thermophilic actinomycetes, was efficient in the degradation of PBAT, but could not be isolated (Šerá et al., 2020b). The relative abundance of microorganisms assigned to *Bacillus* and *Streptomyces* (*Actinomycetes*) also increased after the biodegradation assays, which suggests that they can have a role in PBAT biodegradation. The *Pseudomonas* genus, which includes species previously described as capable of degrading PBAT (*Pseudomonas pseudo alcaligenes*) was significantly reduced (Wallace et al., 2017). PBAT could change the soil microbial community, especially if the soil has more microorganisms capable of taking a role in the biodegradation process (Han et al., 2021). Li et al., (2022) discovered that the soil community richness and diversity decreased with high content of PBAT, comparatively to low PBAT content. In soils with numerous microorganisms capable of degrading PBAT, they will probably grow using PBAT as a carbon source changing the microbial communities. In soils with fewer potential degraders, lower enhancement of PBAT degrading microorganisms occurs, changing less the microbial communities, and this may explain why not many microorganisms significantly change their relative abundance in this soil.

In addition, PHB-degraders were also isolated (Table 4.8). From those, *Streptomyces* sp.*, Fusarium solani, Purpureocillium lilacinum* and *Variovorax* sp. were already described as PHB degraders (Calabia and Tokiwa, 2004; Jeszeová et al., 2018; Kim et al., 2003, 2000; Mergaert and Swings, 1996; Oda et al., 1995). However, isolates 2, 8 and 10 (closely related to *Streptomyces coelicoflavus, Aspergillus insuetus and Clonostachys rosea*, respectively) were never reported to use PHB as carbon and energy source. *Clonostachys rosea* isolated from arctic soils was capable of degrading polycaprolactone (PCL)

(Urbanek et al., 2017). No *Streptomyces coelicoflavus* strains have been identified before with PHB degradation capability. In relation to the genera *Aspergillus*, the species *A. flavus, A. niger, A. ustus* and *A. fumigatus* among others, were described as PHB degraders (Gangurde et al., 2017; Gonda et al., 2000; Kim et al., 2000), but not *A. insuetus*. These microbes have this novel capability and might have contributed to the PHB biodegradation in soil. These results confirm the biodegradation behaviour of the PHB polymer, and the presence in soil of PHB degraders, increasing the existing knowledge about microorganisms with this function.

The biodegradation of the bilayer film detected by the analytical and microscopic methods caused different effects on the prokaryotic and eukaryotic communities. Feng et al., (2022) found that high doses of PHB in soil reduce the relative abundance of *Acidobacteria* due in part to the increased soil pH, and in the present work, *Acidobacteria* also decreased. Microbial functional diversity plays an important part in the degradation of PHB in soil (Dey and Tribedi, 2018). Some bacterial genera with significant differences in their relative abundance (Table S1 - Appendix from Chapter 4), including *Rhodoferax*, *Acidovorax*, *Duganella, Pseudomonas and Acinetobacter* have species capable of degrading PHB but these species were not detected in this work (Colak and Güner, 2004; Kobayashi et al., 1999; Mergaert and Swings, 1996; Suyama et al., 1998). In relation to the eukaryotes (Table 4.6), the genus *Cladosporium*, with several species capable of degrading PHB such as *Cladosporium subcinereum* and *Cladosporium* sp., decreased significantly (Ghosh et al., 2013; Jeszeová et al., 2018; Matavulj and Molitoris, 1992). All of the *Fungi* with significant differences (Table 4.6) increased, and the specie *Talaromyces radicus* has not been previously indicated as PHB degraders but *Talaromyces funiculosus*, *Talaromyces pinophilus,* and *Talaromyces minioluteus* were (Kasuya et al., 2007; Kim et al., 2000). These microorganisms (Table 4.6) are not described in the literature as PBAT or PHB degraders. It is also noteworthy that although in low relative abundance, *Aspergillus ustus* and *Aspergillus oryzae* increased significantly which may indicate their involvement in the biodegradation process since they have been identified in other soils as capable of degrading PHB (Gonda et al., 2000; Sanyal et al., 2006). The genus *Penicillium* has several species identified as PHB degraders, (Mergaert and Swings, 1996; Mergaert et al., 1993), in this work *Penicillium decumbens* ($p < 0.05$) and *Penicillium citrinum,* increased, which may imply their involvement in the degradation. Also, *Streptomyces* sp. and *Bacillus* sp. have several species isolated from soil capable of degrading PHB (Charnock, 2021; Manna et al., 1999; Mergaert and Swings, 1996; Mergaert et al., 1993; Volova et al., 2017). This may explain the increased relative abundance (Table 4.7), which although not statistically significant suggests a possible role of these genera in the degradation process, especially for the PHB layer.

4.5 CONCLUSION

In this work, the bilayer material was degraded by almost 50 % in 6 months. The PHB was most probably completely degraded, and PBAT showed signs of biodegradation during the time course of the experiments, as determined by DSC and ATR-FTIR analysis. The microbial communities changed but not drastically, however the relative abundance of groups of microorganisms related to plastics biodegradation was increased significantly after the incubations, namely those of microorganisms closely related to *Rhodoferax*, *Acidovorax*, *Duganella*, *Pseudomonas* and *Acinetobacter.*

Both PHB and PBAT degraders could be isolated and identified. Several microorganisms were isolated and reported for the first time their ability to biodegrade PHB and PBAT. Regarding PHB degradation, microorganisms assigned to *Streptomyces coelicoflavus, Clonostachys rosea, Aspergillus flavus* could be isolated, and those assigned to *Purpureocillium lilacinum* and *Aspergillus pseudodeflectus* could also be isolated and were found to efficiently biodegrade PBAT at mesophilic conditions. These organisms and their enzymes may be studied and used to improve the biodegradation of the PHB/PBAT in culture media or included in a recycling process for the depolymerization of PBAT.

4.6 REFERENCES

Aarthy, M., Puhazhselvan, P., Aparna, R., George, A. S., Gowthaman, M. K., Ayyadurai, N., ... Kamini, N. R. (2018). Growth associated degradation of aliphatic-aromatic copolyesters by Cryptococcus sp. MTCC 5455. *Polymer Degradation and Stability*, 152, 20–28. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2018.03.021

ASTM D5988-18. (2018). Standard Test Method for Determining Aerobic Biodegradation in Soil of Plastic Materials. *ASTM International.*

Beber, V. C., de Barros, S., Banea, M. D., Brede, M., de Carvalho, L. H., Hoffmann, R., ... Wellen, R. M. R. (2018). Effect of Babassu natural filler on PBAT/PHB biodegradable blends: An investigation of thermal, mechanical, and morphological behavior. *Materials*, 11(5). Retrieved from https://doi.org/10.3390/ma11050820

Biundo, A., Hromic, A., Pavkov-keller, T., Gruber, K., Quartinello, F., Haernvall, K., ... Guebitz, G. M. (2016). Characterization of a poly (butylene adipate- co -terephthalate) - hydrolyzing lipase from Pelosinus fermentans, *Applied Microbiology and Biotechnology,* 100, 1753–1764. Retrieved from https://doi.org/10.1007/s00253-015-7031-1

Calabia, B. P., & Tokiwa, Y. (2004). Microbial degradation of poly(D-3-hydroxybutyrate) by a new thermophilic streptomyces isolate. *Biotechnology Letters*, 26(1), 15–19. Retrieved from https://doi.org/10.1023/b:bile.0000009453.81444.51

Carofiglio, V. E., Stufano, P., Cancelli, N., De Benedictis, V. M., Centrone, D., Benedetto, E. De, ... Demitri, C. (2017). Novel PHB/Olive mill wastewater residue composite based film: Thermal, mechanical and degradation properties. *Journal of Environmental Chemical Engineering*, 5(6), 6001–6007. Retrieved from https://doi.org/https://doi.org/10.1016/j.jece.2017.11.013

Charnock, C. (2021). Norwegian soils and waters contain mesophilic, plastic-degrading bacteria.

Microorganisms, 9(1), 1–18. Retrieved from https://doi.org/10.3390/microorganisms9010094

Colak, A., & Güner, S. (2004). Polyhydroxyalkanoate degrading hydrolase-like activities by Pseudomonas sp. isolated from soil. *International Biodeterioration and Biodegradation*, 53(2), 103–109. Retrieved from https://doi.org/10.1016/j.ibiod.2003.10.006

Dey, S., & Tribedi, P. (2018). Microbial functional diversity plays an important role in the degradation of polyhydroxybutyrate (PHB) in soil. *3 Biotech*, 8(3), 1–8. Retrieved from https://doi.org/10.1007/s13205-018-1201-7

Feng, X., Wang, Q., Sun, Y., Zhang, S., & Wang, F. (2022). Microplastics change soil properties, heavy metal availability and bacterial community in a Pb-Zn-contaminated soil. *Journal of Hazardous Materials*, 424, 127364. Retrieved from https://doi.org/https://doi.org/10.1016/j.jhazmat.2021.127364

Fu, Y., Wu, G., Bian, X., Zeng, J., & Weng, Y. (2020). Biodegradation Behavior of Poly(Butylene Adipate-Co-Terephthalate) (PBAT), Poly(Lactic Acid) (PLA), and Their Blend in Freshwater with Sediment. *Molecules*, 25(17). Retrieved from https://doi.org/10.3390/molecules25173946

Gangurde, N. S., Patil, Y. P., Jain, R., & Sayyed, R. Z. (2017). Poly-β-hydroxybutyrate biodegradation by mixed culture population vis-à-vis single culture population under varying environmental conditions: A new approach. *Indian Journal of Experimental Biology*, 55(5), 311–320. Retrieved from https://nopr.niscpr.res.in/handle/123456789/41722

Ghosh, S. K., Pal, S., & Ray, S. (2013). Study of microbes having potentiality for biodegradation of plastics. *Environmental Science and Pollution Research*, 20(7), 4339–4355. Retrieved from https://doi.org/10.1007/s11356-013-1706-x

Gonda, K. E., Jendrossek, D., & Molitoris, H. P. (2000). Fungal degradation of the thermoplastic polymer poly-β-hydroxybutyric acid (PHB) under simulated deep sea pressure. *Hydrobiologia*, 426(1), 173–183. Retrieved from https://doi.org/10.1023/A:1003971925285

Han, Y., Teng, Y., Wang, X., Ren, W., Wang, X., Luo, Y., ... Christie, P. (2021). Soil Type Driven Change in Microbial Community Affects Poly(butylene adipate- co-terephthalate) Degradation Potential. *Environmental Science and Technology*, 55(8), 4648–4657. Retrieved from https://doi.org/10.1021/acs.est.0c04850

Jeszeová, L., Puškárová, A., Bučková, M., Kraková, L., Grivalský, T., Danko, M., ... Pangallo, D. (2018). Microbial communities responsible for the degradation of poly(lactic acid)/poly(3-hydroxybutyrate) blend mulches in soil burial respirometric tests. *World Journal of Microbiology and Biotechnology*, 34(7), 1–12. Retrieved from https://doi.org/10.1007/s11274-018-2483-y

Jia, H., Zhang, M., Weng, Y., Zhao, Y., Li, C., & Kanwal, A. (2021). Degradation of poly(butylene adipate-co-terephthalate) by Stenotrophomonas sp. YCJ1 isolated from farmland soil. *Journal of Environmental Sciences (China)*, 103, 50–58. Retrieved from https://doi.org/10.1016/j.jes.2020.10.001

kanwal, A., Zhang, M., Sharaf, F., & Chengtao, L. (2022). Screening and characterization of novel lipase producing Bacillus species from agricultural soil with high hydrolytic activity against PBAT poly (butylene adipate co terephthalate) co-polyesters. *Polymer Bulletin*, 79, 10053–10076. Retrieved from https://doi.org/10.1007/s00289-021-03992-4

Kasuya, K., Tezuka, Y., Ishii, N., Yamagata, Y., Shiraki, M., Saito, T., ... Doi, Y. (2007). Molecular Characterization of the Poly(3-hydroxybutyrate) Depolymerase Gene from Penicillium funiculosum. *Macromolecular Symposia*, 249–250(1), 540–544. Retrieved from https://doi.org/https://doi.org/10.1002/masy.200750433

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2010). Biodegradation and hydrolysis rate of aliphatic aromatic polyester. *Polymer Degradation and Stability*, 95(12), 2641–2647. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.07.018

Kim, H. J., Kim, D. Y., Nam, J. S., Bae, K. S., & Rhee, Y. H. (2003). Characterization of an extracellular medium-chain-length poly(3-hydroxyalkanoate) depolymerase from Streptomyces sp. KJ-72. *Antonie van Leeuwenhoek*, 83(2), 183–189. Retrieved from https://doi.org/10.1023/A:1023395527073

Kim, M.-N., Lee, A.-R., Yoon, J.-S., & Chin, I.-J. (2000). Biodegradation of poly(3-hydroxybutyrate), Sky-Green (R) and Mater-Bi (R) by fungi isolated from soils. *European Polymer Journal*, 36, 1677–1685. Retrieved from https://doi.org/10.1016/S0014-3057(99)00219-0

Kobayashi, T., Sugiyama, A., Kawase, Y., Saito, T., Mergaert, J., & Swings, J. (1999). Biochemical and Genetic Characterization of an Extracellular Poly(3-Hydroxybutyrate) Depolymerase from Acidovorax Sp. Strain TP4. *Journal of Environmental Polymer Degradation*, 7(1), 9–18. Retrieved from https://doi.org/10.1023/A:1021885901119

Larsson, M., Markbo, O., & Jannasch, P. (2016). Melt processability and thermomechanical properties of blends based on polyhydroxyalkanoates and poly(butylene adipate-: Co -terephthalate). *RSC Advances*, 6(50), 44354–44363. Retrieved from https://doi.org/10.1039/c6ra06282b

Li, C., Cui, Q., Li, Y., Zhang, K., Lu, X., & Zhang, Y. (2022). Effect of LDPE and biodegradable PBAT primary microplastics on bacterial community after four months of soil incubation. *Journal of Hazardous Materials*, 429(January), 128353. Retrieved from https://doi.org/10.1016/j.jhazmat.2022.128353

Liu, Y., Zhan, Z., Ye, H., Lin, X., Yan, Y., & Zhang, Y. (2019). Accelerated biodegradation of PLA/PHB-blended nonwovens by a microbial community. *RSC Advances*, 9(18), 10386–10394. Retrieved from https://doi.org/10.1039/C8RA10591J

Manna, A., Giri, P., & Paul, A. K. (1999). Degradation of poly(3-hydroxybutyrate) by soil streptomycetes. *World Journal of Microbiology and Biotechnology*, 15(6), 705–709. Retrieved from https://doi.org/10.1023/A:1008980117018

Masood, F., Aziz, M., Haider, H., Shakil, O., Yasin, T., & Hameed, A. (2018). Biodegradation of gamma irradiated poly-3-hydroxybutyrate/sepiolite nanocomposites. *International Biodeterioration and Biodegradation*, 126, 1–9. Retrieved from https://doi.org/10.1016/j.ibiod.2017.09.012

Matavulj, M., & Molitoris, H. P. (1992). Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. *FEMS Microbiology Reviews*, 9(2–4), 323–331. Retrieved from https://doi.org/10.1111/j.1574-6968.1992.tb05854.x

Mergaert, J., & Swings, J. (1996). Biodiversity of microorganisms that degrade bacterial and synthetic polyesters. *Journal of Industrial Microbiology and Biotechnology*, 17(5–6), 463–469. Retrieved from https://doi.org/10.1007/bf01574777

Mergaert, J., Webb, A., Anderson, C., Wouters, A., & Swings, J. (1993). Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. *Applied and Environmental Microbiology*, 59(10), 3233–3238. Retrieved from https://doi.org/10.1128/aem.59.10.3233-3238.1993

Mohanty, S., & Nayak, S. K. (2010). Biodegradable nanocomposites of poly (butylene adipate-co-terephthalate) (PBAT) with organically modified nanoclays. *International Journal of Plastics Technology*, 14(2), 192–212. Retrieved from https://doi.org/10.1007/s12588-010-0018-y

Mousavioun, P., George, G. A., & Doherty, W. O. S. (2012). Environmental degradation of lignin/poly(hydroxybutyrate) blends. *Polymer Degradation and Stability*, 97(7), 1114–1122. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2012.04.004

Muroi, F., Tachibana, Y., Soulenthone, P., Yamamoto, K., Mizuno, T., Sakurai, T., … Kasuya, K. (2017). Characterization of a poly (butylene adipate- co -terephthalate) hydrolase from the aerobic mesophilic bacterium Bacillus pumilus. *Polymer Degradation and Stability*, 137, 11–22. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2017.01.006

Oda, Y., Asari, H., Urakami, T., & Tonomura, K. (1995). Microbial degradation of poly(3-hydroxybutyrate) and polycaprolactone by filamentous fungi. *Journal of Fermentation and Bioengineering*, 80(3), 265–269. Retrieved from https://doi.org/10.1016/0922-338X(95)90827-M

Perz, V., Hromic, A., Baumschlager, A., Steinkellner, G., Pavkov-Keller, T., Gruber, K., ... Guebitz, G. M. (2016). An Esterase from Anaerobic Clostridium hathewayi Can Hydrolyze Aliphatic–Aromatic Polyesters. *Environmental Science & Technology*, 50(6), 2899–2907. Retrieved from https://doi.org/10.1021/acs.est.5b04346

Pietrini, M., Roes, L., Patel, M. K., & Chiellini, E. (2007). Comparative life cycle studies on poly(3-hydroxybutyrate)-based composites as potential replacement for conventional petrochemical plastics. *Biomacromolecules*, 8(7), 2210–2218. Retrieved from https://doi.org/10.1021/bm0700892

Qi, R., Jones, D. L., Liu, Q., Liu, Q., Li, Z., & Yan, C. (2021). Field test on the biodegradation of poly(butylene adipate-co-terephthalate) based mulch films in soil. *Polymer Testing*, 93, 107009. Retrieved from https://doi.org/10.1016/j.polymertesting.2020.107009

Rychter, P., Kawalec, M., Sobota, M., Kurcok, P., & Kowalczuk, M. (2010). Study of aliphatic-aromatic copolyester degradation in sandy soil and its ecotoxicological impact. *Biomacromolecules*, 11(4), 839–847. Retrieved from https://doi.org/10.1021/bm901331t

Sabapathy, P. C., Devaraj, S., Parthipan, A., & Kathirvel, P. (2019). Polyhydroxyalkanoate production from statistically optimized media using rice mill effluent as sustainable substrate with an analysis on the biopolymer's degradation potential. *International Journal of Biological Macromolecules*, 126, 977–986. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.01.003

Sanyal, P., Samaddar, P., & Paul, A. K. (2006). Degradation of Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Some Soil Aspergillus spp. *Journal of Polymers and the Environment*, 14(3), 257–263. Retrieved from https://doi.org/10.1007/s10924-006-0022-7

Šerá, J., Kadlečková, M., Fayyazbakhsh, A., Kučabová, V., & Koutný, M. (2020a). Occurrence and analysis of thermophilic poly(Butylene adipate-co-terephthalate)-degrading microorganisms in temperate zone soils. *International Journal of Molecular Sciences*, 21(21), 1–17. Retrieved from https://doi.org/10.3390/ijms21217857

Šerá, J., Serbruyns, L., De Wilde, B., & Koutný, M. (2020b). Accelerated biodegradation testing of slowly degradable polyesters in soil. *Polymer Degradation and Stability*, 171, 109031. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2019.109031

Šerá, J., Stloukal, P., Jančová, P., Verney, V., Pekařová, S., & Koutný, M. (2016). Accelerated Biodegradation of Agriculture Film Based on Aromatic-Aliphatic Copolyester in Soil under Mesophilic Conditions. *Journal of Agricultural and Food Chemistry*, 64(28), 5653–5661. Retrieved from https://doi.org/10.1021/acs.jafc.6b01786

Soulenthone, P., Tachibana, Y., Muroi, F., Suzuki, M., Ishii, N., Ohta, Y., & Kasuya, K. ichi. (2020). Characterization of a mesophilic actinobacteria that degrades poly(butylene adipate-co-terephthalate). *Polymer Degradation and Stability*, 181, 109335. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2020.109335

Souza, P. M. S., Coelho, F. M., Sommaggio, L. R. D., Marin-Morales, M. A., & Morales, A. R. (2019). Disintegration and Biodegradation in Soil of PBAT Mulch Films: Influence of the Stabilization Systems Based on Carbon Black/Hindered Amine Light Stabilizer and Carbon Black/Vitamin E. *Journal of Polymers and the Environment*, 27(7), 1584–1594. Retrieved from https://doi.org/10.1007/s10924-019-01455-6

Suyama, T., Tokiwa, Y., Ouichanpagdee, P., Kanagawa, T., & Kamagata, Y. (1998). Phylogenetic Affiliation of Soil Bacteria That Degrade Aliphatic Polyesters Available Commercially as Biodegradable Plastics. *Applied and Environmental Microbiology*, 64(12), 5008–5011. Retrieved from

https://doi.org/10.1128/AEM.64.12.5008-5011.1998

Tabasi, R. Y., & Ajji, A. (2015). Selective degradation of biodegradable blends in simulated laboratory composting. *Polymer Degradation and Stability*, 120(February), 435–442. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.07.020

Teixeira, P. F., Covas, J. A., Suarez, M. J., Angulo, I., & Hilliou, L. (2020). Film Blowing of PHB-Based Systems for Home Compostable Food Packaging. *International Polymer Processing*, 35(5), 440–447. Retrieved from https://doi.org/10.3139/217.3985

Tokiwa, Y., & Calabia, B. P. (2007). Biodegradability and Biodegradation of Polyesters. *Journal of Polymers and the Environment*, 15(4), 259–267. Retrieved from https://doi.org/10.1007/s10924-007-0066-3

Trinh Tan, F., Cooper, D. G., Marić, M., & Nicell, J. A. (2008). Biodegradation of a synthetic co-polyester by aerobic mesophilic microorganisms. *Polymer Degradation and Stability*, 93(8), 1479–1485. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2008.05.005

Urbanek, A. K., Rymowicz, W., Strzelecki, M. C., Kociuba, W., Franczak, Ł., & Mirończuk, A. M. (2017). Isolation and characterization of Arctic microorganisms decomposing bioplastics. *AMB Express*, 7(1), 148. Retrieved from https://doi.org/10.1186/s13568-017-0448-4

Volova, T. G., Prudnikova, S. V, Vinogradova, O. N., Syrvacheva, D. A., & Shishatskaya, E. I. (2017). Microbial Degradation of Polyhydroxyalkanoates with Different Chemical Compositions and Their Biodegradability. *Microbial Ecology*, 73(2), 353–367. Retrieved from https://doi.org/10.1007/s00248-016-0852-3

Wallace, P. W., Haernvall, K., Ribitsch, D., Zitzenbacher, S., Schittmayer, M., Steinkellner, G., … Birner-Gruenberger, R. (2017). PpEst is a novel PBAT degrading polyesterase identified by proteomic screening of Pseudomonas pseudoalcaligenes. *Applied Microbiology and Biotechnology*, 101(6), 2291–2303. Retrieved from https://doi.org/10.1007/s00253-016-7992-8

Wang, H., Wei, D., Zheng, A., & Xiao, H. (2015). Soil burial biodegradation of antimicrobial biodegradable PBAT films. *Polymer Degradation and Stability*, 116, 14–22. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2015.03.007

Weng, Y. X., Jin, Y. J., Meng, Q. Y., Wang, L., Zhang, M., & Wang, Y. Z. (2013). Biodegradation behavior of poly(butylene adipate-co-terephthalate) (PBAT), poly(lactic acid) (PLA), and their blend under soil conditions. *Polymer Testing*, 32(5), 918–926. Retrieved from https://doi.org/10.1016/j.polymertesting.2013.05.001

Witt, U., Einig, T., Yamamoto, M., Kleeberg, I., Deckwer, W. D., & Müller, R. J. (2001). Biodegradation of aliphatic-aromatic copolyesters: Evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. *Chemosphere*, 44(2), 289–299. Retrieved from https://doi.org/10.1016/S0045-6535(00)00162-4

Wu, C. S. (2012). Characterization of cellulose acetate-reinforced aliphatic-aromatic copolyester composites. *Carbohydrate Polymers*, 87(2), 1249–1256. Retrieved from https://doi.org/10.1016/j.carbpol.2011.09.009

Zumstein, M. T., Rechsteiner, D., Roduner, N., Perz, V., Ribitsch, D., Guebitz, G. M., … Sander, M. (2017). Enzymatic Hydrolysis of Polyester Thin Films at the Nanoscale: Effects of Polyester Structure and Enzyme Active-Site Accessibility. *Environmental Science & Technology*, 51(13), 7476–7485. Retrieved from https://doi.org/10.1021/acs.est.7b01330

CHAPTER 5.

IMPACT OF SOIL TEMPERATURE, WATER HOLDING CAPACITY AND CARBON:NITROGEN RATIO ON PHB/PBAT – AN EXPERIMENTAL DESIGN APPROACH

5.1 INTRODUCTION

Most biodegradation studies usually evaluate the effects of a single factor at a time by changing the value of one variable and keeping the other variables constant (Pischedda et al., 2019). However, in many areas, experimental design techniques have been employed in product and process optimization aiming at minimizing costs and saving time, as well as maximizing productivity and product quality. Beyond reducing the number of trials, experimental design analyses factors simultaneously and allows the optimization of more than one response at a time (Rodrigues and Iemma, 2014). Furthermore, the application of experimental design strategies (e.g., central composite rotational design - CCRD) results in the mathematical modulation of the assessed scenario so that estimations can be made. This approach can be used in research, development and production, for example, to optimize culture media (Tarrahi et al., 2020).

There is an increased necessity to understand the impact of several parameters in the biodegradation process of the different solutions since the biodegradation behaviour can vary depending on the type of polymer and exposure conditions. The effect of temperature is normally considered to be significant during the biodegradation of polymers in soil, even though it can affect distinctly different polymers. Nishide et al. (1999) found that 52 °C was an optimal temperature for the degradation of polybutylene succinate adipate (PBSA) in soil, but the effect of temperature was not significant with poly(ε-caprolactone) (PCL) and polybutylene succinate (PBS). At lower temperatures, below 10 °C, it is challenging to have significant degradation (Briassoulis and Mistriotis, 2018). Since the mesophilic microorganism growth range of temperature is 10 to 45 °C, values outside this range may in fact limit biodegradation (Pischedda et al., 2019). Mergaert et al. (1993) discovered that the degradation rate in soils (i.e., laboratory testing) of PHB was enhanced at higher temperatures (40 °C). Nevertheless, Šerá et al. (2020a) found that PHA degraded faster at 25 °C than at 37 °C in two laboratory studies. These contradictory results may be due to the higher biomass build-up and consequent carbon retention, according to the authors (Šerá et al., 2020a). PBAT degradation is much lower in soil at medium temperatures, being faster in compost settings (i.e., around 55 °C) (Han et al., 2021; Kijchavengkul et al., 2010a).

Another relevant parameter is water, which is considered a crucial factor for the development of microorganisms. Very low water content, i.e., 10 % or 20 %, results in no disintegration for more than two months and slower in the other tested conditions (Briassoulis et al., 2020). Other relevant factor to be considered is the nutrient content of the soil and, the most important nutrient for biodegradation is nitrogen since it is required for the growth of microorganisms due to its expressive presence in nucleic

acids and amino acids (Briassoulis et al., 2020). Hoshino et al. (2001) showed through a study conducted in several soil sites that the degree of degradation of several polymers was more correlated with the total nitrogen content in several sampling sites than with the soil carbon content. According to the authors, this may be related to the plastic composition (carbon, hydrogen, oxygen), i.e., it lacks nitrogen. This limitation can favour fungi, since they can provide nitrogen from nutrient-rich soil areas to cells positioned on the plastic surface through the cytoplasmatic current in their hyphal network (Frey et al., 2000). Nitrogen-fixing bacteria may also be important since they can provide nitrogen to fungi capable of decomposing plastics through the production of enzymes (Purahong et al., 2021). This type of factors may impact directly the soil community composition and increase or decrease the polymer biodegradation.

In this chapter the effect of temperature, soil moisture-holding capacity (MHC), and nitrogen on the biodegradation of PHB/PBAT bilayer films in soil was studied, by applying the central composite rotational design (CCRD), to test each variable effect and the interactions between them. A prediction model was developed and tested, to estimate the PHB/PBAT film biodegradability.

5.2 MATERIAL AND METHODS

5.2.1 Soil collection and characterization

The soil was sampled at the University of Minho (Campus of Gualtar, Braga, Portugal). All the procedures were done exactly as described in section 3.2.1 Soil media of Chapter 3. The only difference was that the soil was sieved to 1 mm. The physicochemical characteristics are presented in Table S1 (Appendix from Chapter 5). In total 19 trials were conducted using combinations of the 3 factors tested: temperature, MHC, and the C:N ratio (nitrogen added to the soil in relation to the carbon existing in the plastic). In the end, a validation trial was conducted using the best combination possible. The two samplings occurred in October 2020 and July 2021. The sampling of the validation trial occurred in July 2022.

5.2.2 Biodegradation assays

Biodegradation experiments were performed using 38 reactors (3 L wide-mouth glass jars), inoculated with the soil (250 mg). The system was set up according to section 3.2.2 Biodegradation experiments set-up. The plastic used (PHB/PBAT 55/45 in weight % bilayer film) and the reference material (cellulose) were the same as described in section 4.2.2 Biodegradation experiments set-up. Different amounts of

the blend and cellulose paper were added to the bioreactors to obtain an equivalent mass of organic carbon, according to Table 5.1.

Table 5.1 Weight and total carbon content of the materials tested. All experiments were performed in triplicate

Bioreactor	Test Material	Carbon (%)	mass (mg)	Total carbon (mg)
PHB/PBAT Film	PHB (45 %) PBAT (55 %)	60.9*1	498.8 – 501.7	303.7 – 305.50
Cellulose filter paper	Cellulose (100 %)	44.0*2	684.4 – 686.2	304.2 – 305.0

*1-Determined by elemental analysis. *2 – Determined taking into account its chemical composition ($C_6H_{10}O_5$)

5.2.3 Design of experiments (DoE)

5.2.3.1. Experiments

The variables tested using the DoE were the soil temperature (9 - 45 °C MHC (60 -100 %), and the C:N ratio (nitrogen added to the soil in relation to the carbon existing in the plastic) (0 - 40), as specified in Table 5.3. The biodegradation was determined through the analysis of the accumulated CO_2 (using the same method described before and based on the ASTM D5988-18 (2018)). A three-variable CCRD (2^3) was used, with five repetitions of the central point (0) and six axial points. The addition of axial points (-1.68 and +1.68) enables the adjustment of the data to a second-order model and, consequently, confirms the existence of curvature (Rodrigues and Iemma, 2014). Variables and levels are demonstrated in Table 5.2.

Table 5.2 Variables and levels used for the CCRD

Independent Variables	-1.68	-1	0	1	1.68
C:N ratio	0	8.1	20.0	31.9	40.0
Temperature (°C)	9.0	16.3	27.0	37.7	45.0
MHC (%)	60.0	68.0	80.0	92.0	100.0

In total, 19 assays were performed, as indicated in Table 5.3. Since the assays were conducted in two parts, with two soils, 5 assays for the central point were included instead of 3 (i.e., 3 for the first soil and 2 for the second soil). The soils were collected from the same site and presented similar properties, although slight differences may be possible, for example at the microorganism community's composition

level. The DoE data were analysed using Protimiza Experimental Design Software, Brazil (https: //experimental-design.protimiza.com.br/).

Table 5.3 CCRD trials performed, with the temperature, C:N ratio and MHC conditions specified for each trial

Trials	C:N ratio	T (°C)	MHC (%)
1	8.1 (-1)	16.3 (-1)	68.1 (-1)
2	31.9 (1)	16.3 (-1)	68.1 (-1)
3	8.1 (-1)	37.7 (1)	68.1 (-1)
4	31.9 (1)	37.7 (1)	68.1 (-1)
5	8.1 (-1)	16.3 (-1)	91.9 (1)
6	31.9 (1)	16.3 (-1)	91.9 (1)
7	8.1 (-1)	37.7 (1)	91.9 (1)
8	31.9 (1)	37.7 (1)	91.9 (1)
9	0.0 (-1.68)	27.0 (0)	80.0 (0)
10	40.0 (1.68)	27.0 (0)	80.0 (0)
11	20.0 (0)	9.0 (-1.68)	80.0 (0)
12	20.0 (0)	45.0 (1.68)	80.0 (0)
13	20.0 (0)	27.0 (0)	60.0 (-1.68)
14	20.0 (0)	27.0 (0)	100.0 (1.68)
15	20.0 (0)	27.0 (0)	80.0 (0)
16	20.0 (0)	27.0 (0)	80.0 (0)
17	20.0 (0)	27.0 (0)	80.0 (0)
18	20.0 (0)	27.0 (0)	80.0 (0)
19	20.0 (0)	27.0 (0)	80.0 (0)

A validation trial was further performed to confirm and assess the quality of the DoE. The values of the validation trial, where the most significant for each parameter. In cases where the parameters were not relevant, the central point was selected.

5.2.3.2. PHB/PBAT film biodegradation prediction

The biodegradation of PHB/PBAT films was assessed for 6 months, and a mathematical modulation of the PHB/PBAT films biodegradation for each month of the assay was made. A prediction model was developed by correlating different physicochemical characteristics of the soil with the biodegradation time of the film. For this purpose, a regression model (Equation 1) was adjusted using the experimental setup of the previous CCRD (Table 5.3).

$$y = \alpha * \ln(t) + \beta \quad (1)$$

Where y corresponds to the films' biodegradability and t corresponds to time (in months). It was also possible to observe that the two coefficients of the regression model described in Equation 1 are related to the physicochemical characteristics of the soil. As such, a similar CCRD was used to determine the equations needed to calculate the coefficients α (responsible for the vertical stretch of the curve) and β (responsible for the horizontal displacement of the curve).

These equations were used to predict the biodegradation of the material tested in this work in similar soils, with a previous characterization of the soil in relation to three factors tested: temperature, MHC and nitrogen added concerning the carbon in the sample.

5.2.4 Analytical methods

To evaluate the effect of the biodegradation on film properties in the validation assay, the PHB/PBAT films were analysed before and after the incubations to verify the effects on the functional groups using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Differential Scanning Calorimetry (DSC) to evaluate the changes in the thermal properties. These two techniques were used as described in section 3.2.3 Analytical and microscopic methods.

5.2.5 Statistical analysis

The DSC values the analysed as described in section 3.2.7 Statistical analysis of Chapter 3. The principal component analysis (PCA) conducted on the experimental trials of the CCRD was also performed using the OriginPRO 2019b statistical program.

5.3 RESULTS

5.3.1 Design of experiments for the biodegradation assay

The physicochemical properties of the soil were within the recommended ranges by the ASTM D5988-18 (2018), namely the pH was in the range 6 - 8 in all trials as indicated in Table 5.4. More importantly, the differences were slight, which contributed to the maximum homogeneity possible between the trials.

Table 5.4 Physicochemical properties of the soil. All experiments were performed in triplicate

Analysis	1° Soil	2° Soil	Validation Soil
Total dry solids (%)	92.45	91.41	92.53
Volatile solids (%)	7.55	8.59	7.47
pH	6.53	6.52	7.14
Total organic carbon amount (%)	2.71	3.82	3.98
Total nitrogen amount (%)	0.15	0.18	0.23
Carbon/nitrogen ratio (C:N)	18.07	21.22	17.3
Moisture holding capacity	19.43	19.26	18.44

*1 –Determined using the SM 2540 G. (1998), *2 – Determined using the ASTM D1293 (2018), *3 –Determined by elemental analysis, *4 – Determined using the ASTM D425 (2017).

PHB/PBAT films were biodegraded in all conditions tested (Table 5.5).

Table 5.5 Percentage of biodegradation of the PHB/PBAT films during 6 months for each, DoE trial. The validation was performed in triplicate

Trials	C:N ratio	T (°C)	MHC (%)	Biodegradation (%) Months					
				1	2	3	4	5	6
1	8.1 (-1)	16.3 (-1)	68.1 (-1)	5.3	14.7	18.4	27.3	33.2	38.4
2	31.9 (1)	16.3 (-1)	68.1 (-1)	1.9	6.4	14.7	22.8	27.2	32.9
3	8.1 (-1)	37.7 (1)	68.1 (-1)	34.4	41.3	42.1	46.1	48.9	51.5
4	31.9 (1)	37.7 (1)	68.1 (-1)	32.2	42.6	46.5	49.0	54.4	59.3
5	8.1 (-1)	16.3 (-1)	91.9 (1)	4.5	19.7	26.4	31.9	35.5	40.6
6	31.9 (1)	16.3 (-1)	91.9 (1)	2.8	9.7	18.5	31.2	34.0	39.5
7	8.1 (-1)	37.7 (1)	91.9 (1)	31.1	36.5	38.2	43.9	52.5	57.6
8	31.9 (1)	37.7 (1)	91.9 (1)	27.3	43.7	45.3	47.7	53.5	60.7
9	0.0 (-1.68)	27.0 (0)	80.0 (0)	6.5	23.1	27.3	32.4	40.7	42.6
10	40.0 (1.68)	27.0 (0)	80.0 (0)	9.0	27.0 (0)	31.8	36.7	42.4	48.0
11	20.0 (0)	9.0 (-1.68)	80.0 (0)	5.3	14.8	18.1	21.5	25.1	28.7
12	20.0 (0)	45.0 (1.68)	80.0 (0)	12.4	38.4	51.7	55.5	57.1	58.5
13	20.0 (0)	27.0 (0)	60.0 (-1.68)	16.8	25.1	30.8	37.8	43.3	45.1
14	20.0 (0)	27.0 (0)	100.0 (1.68)	14.5	27.1	35.3	37.8	40.2	44.9
15	20.0 (0)	27.0 (0)	80.0 (0)	11.8	23.2	30.7	36.4	45.3	48.2
16	20.0 (0)	27.0 (0)	80.0 (0)	12.5	25.2	34.6	33.7	40.0	43.7
17	20.0 (0)	27.0 (0)	80.0 (0)	14.6	26.3	29.6	33.2	41.0	46.8
18	20.0 (0)	27.0 (0)	80.0 (0)	14.7	25.3	32.7	37.4	43.3	47.6
19	20.0 (0)	27.0 (0)	80.0 (0)	15.3	24.2	28.9	34.0	40.4	46.0
Validation (Triplicates)	20	37.7	80	22.8	36.5	42.0	46.0	51.1	55.3

Biodegradation percentages varied between 28.65 % and 60.73 %, which corresponded to the conditions of trial 11 (lowest temperature) and 8, respectively (Table 5.5). The 19 trials performed for the DoE demonstrated (Table 5.5) that the increased temperature resulted in an increased biodegradation. The PCA analysis (Figure 5.1) also supported this conclusion. In the trials where 37.7 °C or higher temperatures were used (trials 3, 4, 7, 8 and 12) the biodegradation reached > 51 % after 6 months, while with the lower temperatures tested the maximum biodegradation obtained was of 48. 2 % (trial 15). With the two lowest temperatures tested (9 and 16.3 °C), the biodegradation never passed the 41 % barrier (trial 5).

All the parameters which were not significant were eliminated from the DoE equations. The results of the CCRDs demonstrated that the temperature was the only parameter tested that was significant in all the CCRDs performed each month (Table 5.6). The humidity was just significant in the sixth month, and in interaction with temperature in the third and fourth months. The C:N ratio was only significant in interaction with the temperature in all months, except for the first one (Table 5.6).

Table 5.6 DoE equations for each month. x_1 – C:N ratio, x_2 – temperature, x_3 – MHC

Months	DoE Equations	
1	$Y_1 = 14.38 + 8.98\ x_2$	$R^2 = 52.86$ %
2	$Y_2 = 26.18 + 11.07\ x_2 + 2.93\ x_1\ x_2$	$R^2 = 89.24$ %
3	$Y_3 = 31.66 + 11.07\ x_2 + 2.88\ x_1\ x_2 - 2.09\ x_2\ x_3$	$R^2 = 94.67$ %
4	$Y_4 = 36.15 + 9.56\ x_2 + 0.99\ x_2^2 + 1.50\ x_1\ x_2 - 2.08\ x_2\ x_3$	$R^2 = 95.89$ %
5	$Y_5 = 41.99 + 9.75\ x_2 + 1.74\ x_1\ x_2$	$R^2 = 95.45$ %
6	$Y_6 = 46.51 + 9.37\ x_2 + 1.17\ x_3 + 2.19\ x_1\ x_2$	$R^2 = 92.90$ %

5.3.2 A multivariate perspective

In the PCA analysis (Figure 5.1) the principal component 1 (PC1) and principal component 2 (PC2) explain 71.52 % and 11.55 % of the data variability, respectively. Consequently, it is important to mention that the horizontal distance between the different points is significantly more important than their vertical displacement. As such, it is possible to see in Figure 5.1 the categorization of the experimental trials according to the experimental temperature, with the conditions corresponding to the higher temperatures at the right (45 and 37 °C), the intermediate temperature (27 °C) at the center, and the lower temperatures at the left (9 and 16 °C). The PCA clearly shows that the temperature was the most important factor since it is mainly represented by PC1. Moreover, it is also possible to confirm that in fact, higher temperatures promote a higher degradation of the PHB/PBAT films since the loadings

indicate a correlation between the experimental temperature and the biodegradation in each month. It was also possible to confirm that both humidity and C:N did not significantly impact the biodegradation of the polymers due to their orthogonal placement when compared to the temperature and biodegradation in each month.

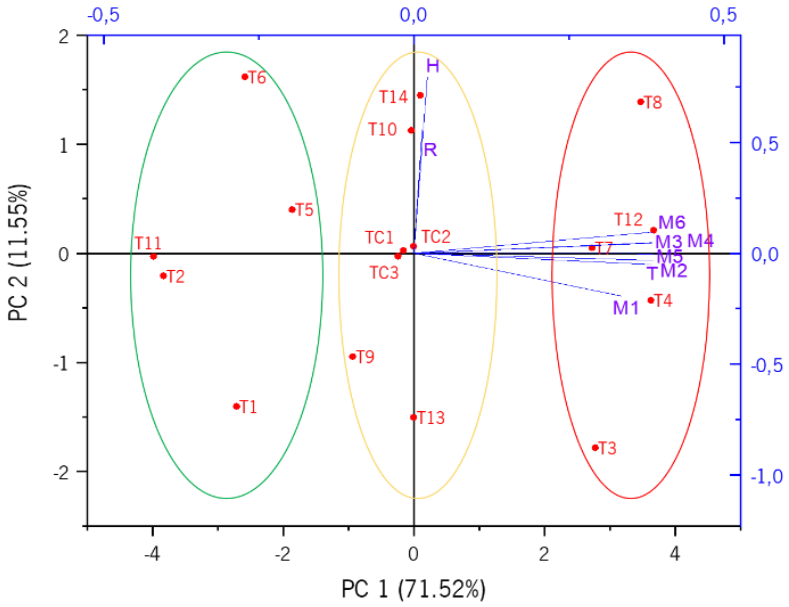

Figure 5.1 Principal component analysis of the biodegradation experimental trials (represented by T1-T14, and TC for the central points) where H represents the soil's humidity, R corresponds to the C:N ratio, T corresponds to the temperature and M(1-6) to the month.

5.3.3 Analytical analysis of the validation trial

The analytical techniques revealed that most of the biodegradation during the validation trial (C:N ratio of 20, 37.7 °C temperature and 80 % of soil holding capacity), was due to the PHB layer. The ATR-FTIR demonstrated that the PHB layer was the main contributor to biodegradation. No PHB spectra were present on the residues at the end of the assay. The PBAT layer presented clear signs of serious biodegradation with a marked reduction in all peaks, such as the aromatic (1267 cm^{-1}) and the carbonyl absorbance peak (1709 cm^{-1}) (Figure 5.2). These reductions indicate biodegradation (Qi et al., 2021). Several small new peaks developed (e.g., 3937 3872, 3745, 3694, 3617, 3289 cm^{-1}) some of them related to the formation of free O-H and hydroxyl and carboxylic, linked to the scission in the main chain resulting from the hydrolysis or the action of enzymes (Sabapathy et al., 2019).

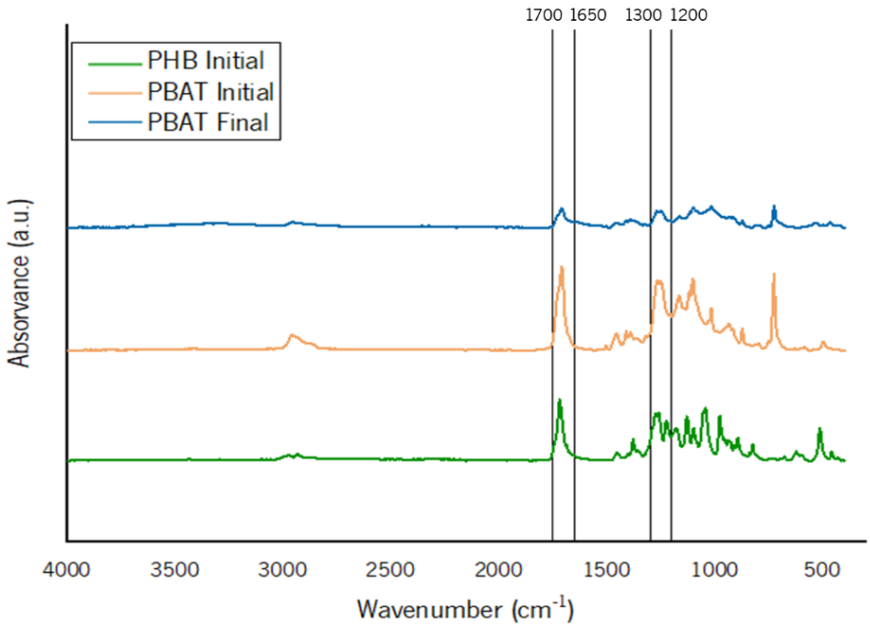

Figure 5.2 Infrared spectrums of the PHB/PBAT films before and after soil biodegradation (validation trial).

The DSC analysis also revealed that the PHB disappeared from the sample and all the parameters were significantly affected ($p < 0.05$), except for the melting temperature onset. The increased crystallinity demonstrated the preference for biodegradation of the amorphous areas of the PBAT layer (Kijchavengkul et al., 2010a). The PBAT melting temperature increased significantly as indicated in Table 5.7, indicating the biodegradation and hydrolysis of the aliphatic part which led to an improved crystalline structure with fewer defects (Fu et al., 2020).

Table 5.7 The thermal properties of the samples before and after biodegradation were determined by DSC. All experiments were performed in triplicate

Samples	T_{m^1}(°C)	Δh^1 (J/g)	T_m Onset^1 (°C)	T_{c^1} (°C)	X_{c^1} (%)	T_{m^2}(°C)	Δh^2 (J/g)	T_m Onset^2 (°C)	T_{c^2} (°C)	X_{c^2} (%)
Initial PHB/PBAT Film	171.30 ± 0.32	34.10 ±3.30	165.92 ± 0.72	112.03 ± 0.18	23.35 ± 2.26	118.1 ± 0.2	4.5 ± 0.8	98.2 ± 2.8	67.9 ± 0.4	3.9 ± 0.7
Final PHB/PBAT Film						123.8 ± 1.4	12.5 ± 1.4	100.8 ± 1.6	73.7 ± 1.3	10.9 ± 1.2

It is also noteworthy that in the trials with the temperature in the range recommended by the standard method (20-28 °C), the control biodegradation (cellulose) was equal or higher than the > 70 % needed for the assay to be considered valid. The same was observed at 16.3 °C and 45 °C, however at 9 °C

and 37.7 °C this condition was not achieved (Table S1 - Appendix from Chapter 5). This may happen because some temperatures favour the cellulose-degrading microorganisms or there are some differences in the communities in each reactor, and thus different biodegradation potential may be expected. Furthermore, the higher biomass build-up and consequent carbon retention may happen at higher temperatures (in this case 37.7 °C) (Šerá et al., 2020a). This could explain the lower biodegradation observed in some trials using this temperature.

5.3.4 Validation and estimation of the biodegradation of PHB/PBAT films

Estimating the biodegradation of PHB/PBAT films could be a very useful strategy to determine the biodegradation of PHB/PBAT films in soils with a previous physicochemical characterization. However, it is important to characterize the obtained results in the DoE. For this purpose, a validation assay was used, within the tested parameters' range, with a different combination of experimental conditions, i.e., a C:N ratio of 20, at 37.7 °C and an MHC of 80 %. The results of the validation trial are depicted in Figure 5.3.

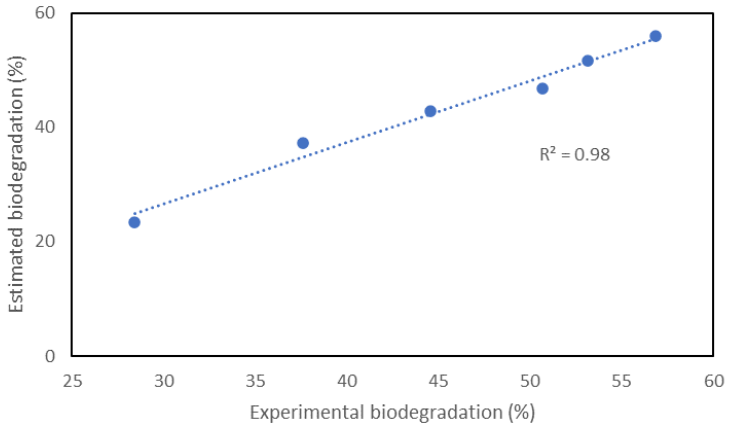

Figure 5.3 Comparison between the experimental validation trial and the prediction model using monthly equations.

Figure 5.3 shows that the estimated and experimentally obtained biodegradation values in the validation trial have a 0.98 correlation coefficient. A logarithmic regression model was further used to estimate the biodegradation of PHB/PBAT films by correlating the physicochemical characteristics of the soil with the biodegradation at a specific time. Thus, the equations to calculate the model's coefficients were obtained using a CCRD and the results are expressed in Equations 2 and 3.

$$\alpha = 17.34 + x_2 + 0.13{x_2}^2 + 0.37x_3 + 0.07x_1x_2 + 0.42x_2x_3 \quad (2)$$

$$\beta = 13.80 + x_1 + 9.97x_2 + 0.02{x_2}^2 - 0.21x_3 + 1.43x_1x_2 + 0.79x_2x_3 \quad (3)$$

Where x_1 corresponds to the C:N ratio, x_2 corresponds to the soil temperature and x_3 corresponds to the MHC. This way, the logarithmic model (Equation 1) can be further used to estimate the biodegradation of PHB/PBAT films through time and the results are depicted in Figure 5.4.

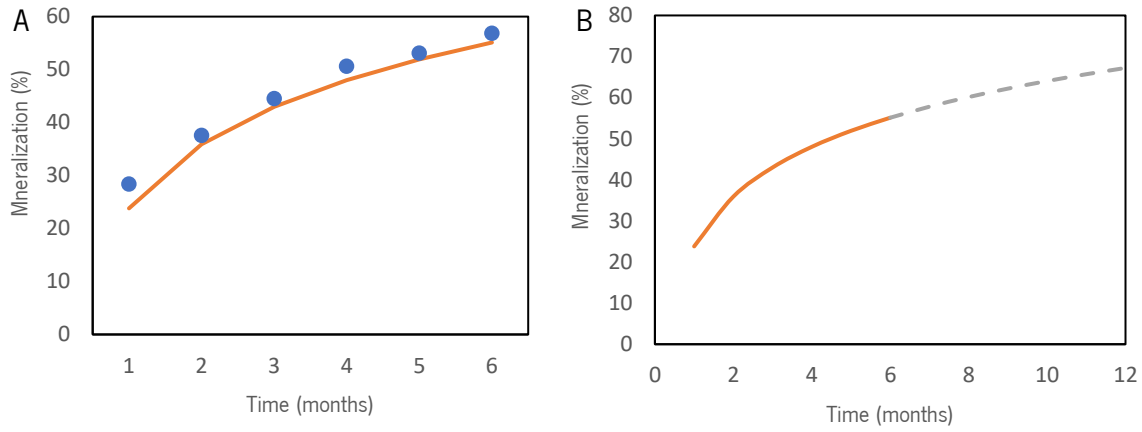

Figure 5.4 A) Comparison between the experimental validation trial (dots) and the prediction model using the final equation (line). B) Prediction using the validation parameters beyond the 6 months tested (dashed line).

It is possible to observe in Figure 5.4A that the prediction model (Equation 1) can predict with high accuracy the biodegradation of PHB/PBAT films since the correlation between the estimated and experimental results is of ca. 0.99, which can be considered a high correlation, considering that this process is mainly mediated by the presence of microorganisms which can present a high variability. The correlation is 0.93 between all the experimental values indicated in Table 5.5 and the estimations of the model under the same conditions.

5.4 DISCUSSION

Considering the values recommended by the standard method (ASTM D5988-18, 2018) for the C:N ratio (nitrogen added to the soil in relation to the carbon in the test specimen), although 0 and 40:1 were used, this parameter had no clear impact on the final biodegradation percentages, not being significant in any equation (Table 5.5). Kijchavengkul et al. (2010a) found that compost with a higher C:N ratio than the recommended, resulted in less polymer biodegradation. It has been observed in other work that the carbon input presented by the PHBV degradation may cause the microbial immobilization of nitrogen sources to sustain microbial growth, since the PHBV degradation increased by 45 % the microbial biomass

nitrogen and reduced the dissolved organic nitrogen by 66 %. (Zhou et al., 2021). In this work, although this parameter was tested, the addition of nitrogen did not have a significant impact on the biodegradation of the PHB/PBAT films, probably because the carbon of the polymer and nitrogen added were not sufficient to significantly shift the C:N ratio existing in the soil, which explains the similar biodegradation between all the C:N ratio conditions tested. The bilayer material tested is only composed of C, O, and H atoms which means that the potential microbial degraders need to obtain N from the surrounding soil to grow. In nitrogen-poor soils, the biodegradation of polymers may be limited by the deficit of this nutrient in relation to the added carbon. The lack of sufficient nitrogen may favour the action of fungi, which need relatively lower amounts of nitrogen for growing and also probably heterotrophic nitrogen-fixing bacteria (Güsewell and Gessner, 2009; Hodge et al., 2000). This insufficiency may condition the composition of the soil community, originating different biodegradation outcomes. In these biodegradation tests, nitrogen was not limiting as shown by the close biodegradation values between the intermediate (20) C:N ratio (43.7 % - 48.2 %, trials 14 to 19) and the lowest (0) and highest (40) C:N ratio tested, 42.6 % (trial 9) and 48 % (trial 10) respectively.

Regarding humidity, it is recommended an 80 to 100 % MHC (ASTM D5988-18, 2018). However, lower values were used in this study and this parameter had no significant impact on biodegradation in the tested range (from 60 to 100 %) producing similar results, only differing by 0.2 % (trials 13 and 14). Water absorption can interact with the polymer structure causing hydrolytic degradation (abiotic process), resulting in the cleavage of ester bonds (Camani et al., 2021). In the case of PBAT, the absorption of water in the amorphous regions of the aromatic polyester can cause swelling and stress resulting in microcavitation (Kijchavengkul et al., 2010b). The degradation of PHB is also higher in soils with more water content than in soils with less water (Lim et al., 2005). The moisture content can thus influence the degradation. Products with several materials, such as PHAs can even present a superior water absorption percentage, for example, PHBHHx/KF presented a higher water absorption percentage and a higher percentage of weight loss during a soil degradation study when compared to the PHBHHx (Joyyi et al., 2017). Briassoulis and Mistriotis (2018) used the same standard methods, although to test lubricants. The authors recommended a range of 60 % to 100 % of the soil water holding capacity and an ideal value of 80 % for the greatest possible disintegration rate. This result may explain why the soil's moisture in the present biodegradation assay did not present significant differences in the range used, i.e., it was already within the optimal range.

It is expected that environmental parameters have an immediate effect on biodegradable plastic build-up after release (accidental or intentional) since they directly influence the plastic biodegradation rates. For modelling pollutant degradation half-lives in chemical fate assessment and exposure models used for risk assessment purposes, the temperature is used as a predictor variable (Matthies and Beulke. 2017). The range of temperatures recommended by the standard methods is between 20 °C and 28 °C. The increased temperature tested, namely 37.7 and 45 °C, increased the biodegradation by 10.8 % and 11.5 % respectively. This observation could be due to the abiotic degradation or/and the enhanced activity of some microorganisms in these conditions. When neat PHAs were tested in other works, the temperature also was an important factor. Kim et al. (2000) demonstrated that the degradation of PHB was higher in different soils at 37 °C than at 28 °C, although the worst temperature for degradation was 60 °C. These differences were caused by the activity of the soil microbial community, which is strongly influenced by the temperature of the environment. PBAT degradation is also faster at composting temperatures (i.e., 55 °C) (Han et al., 2021; Kijchavengkul et al., 2010a). By analysing the individual polymers in relation to temperature, it was expected that higher temperatures should increase or at least accelerate the biodegradation process. This was confirmed by the PCA analysis (Figure 5.1). Temperature can effectively disturb chemical and biochemical reactions and influence the taxonomic structure and metabolic activities of the communities (Schulte. 2015). Usually, the biodegradation process increases with temperature increase until it reaches a plateau (i.e., where it is included in the thermal optimum) and finally, as the temperature increases more, the biodegradation decreases, because enzymes start to denature, and the biodegradation outcome becomes ineffective (Schulte et al., 2011). This effect explains the small difference in the biodegradation between the 37.7 °C and 45 °C conditions, which was only 1.3 % more, for the higher temperature.

For this specific bilayer film, an increase in temperature was beneficial under the tested soil characteristics. Although with 45 °C the biodegradation was slightly better, a temperature around 37 °C should be used to maximize soil biodegradation since it requires less energy to be maintained. Furthermore, under this temperature condition (i.e., 37 °C) the maximum temperature of 45 °C for the growth of mesophilic microorganisms is avoided, and the microbiome is probably not significantly shifted. The microbiome is normally efficient in the degradation of neat PHAs in temperatures between 20 - 30 °C. PBAT is compostable and several thermophilic organisms have been identified, with higher temperatures benefiting the degradation of this polymer (Šerá et al., 2020b). The abiotic effect at these temperatures may be also higher (Šerá et al., 2020b). This effect in combination (or not) with the more effective action of the PBAT degrading microorganisms caused the enhanced degradation in this work.

Šerá et al. (2020a) found that the differences detected in the bacterial community between the temperatures 25 °C and 37 °C were not relevant for the slow-degrading polymers (PBAT/PLA) but affected the faster degrading polymers (PHA and PBS), which supported mostly the fast-growing microorganisms. However, some changes occurred in the fungal communities with more thermophilic than mesophilic species, which may explain the faster biodegradation of the PBAT/PLA powder. The analysis of the communities in different conditions can be very interesting to test in future works; since the bilayer material used is composed of PHB (a biodegradable material with many soil organisms capable of degrading it) and PBAT (which although compostable), it has not many organisms identified as able to degrade it. An evaluation of the residues (e.g., microplastics) and metabolites should also be conducted, to analyse further the environmental impact.

The biodegradation performance in the validation trial was among the best results because the temperature chosen was within the most significant range and for the other parameters the central point was selected. The results indicate that the PHB fraction was more biodegraded (Table 5.7 and Figure 5.2), as described earlier in chapter 4. The PHB polymer was no longer detected after the assays in the film by the ATR-FTIR and DSC analyses. The PBAT was less degraded but also contributed to the CO_2 evolution, which was confirmed by the development of small peaks related to the scission in the main chain, and the PBAT increased crystallinity related to the degradation of the amorphous regions.

The 0.98 correlation coefficient (Figure 5.3) obtained for the estimated and experimentally biodegradation values (Validation trial) indicates that the DoE approach can be used, not only to optimize a predetermined process (which is the most common application of DoE) but it can also be used to modulate the biodegradation of bioplastics using fewer trials. The quality of the prediction model (Figure 5.4A) enables the possibility to predict the soil biodegradation of these films at any given temperature, MHC and C:N ratios, within the tested range. For example, in Braga (the area where the soil was sampled) the annual temperature of soils at a plough depth in 2021 was between 1.3 °C and 26.2 °C (IPMA - Instituto Português do Mar e da Atmosfera (personal communication)), being the average soil temperature of 14.4 °C. Considering the holding capacity of the soil, it is not inferior to the threshold tested (60 %) and no nitrogen is added to the soil in the case of the release of the films to the environment, and the type of soil is similar to the one used to produce the model, the model estimates that the total degradation will reach in 6 months, 35.74 %. Instead of the temperature annual average, the value of any parameter can be changed over time, during the prediction, using the real values measured and following the seasonal changes. Using the same model beyond the 6 months tested (Figure 5.4B), it is possible to consider that

the bilayer film would reach almost 70 % biodegradation in 12 months. However, these types of extrapolations beyond 6 months should only be considered indicative since the model was built using tested (experimental) values for 6 months.

This model is a tool that considers several variables, nonetheless, it has several simplifications and represents an approximation of the biodegradation and not an exact prediction. For example, UV light can interact with polymers. The photodegradation can cause crosslinking within the film due to the recombination of the produced free radicals from Norrish I. Kijchavengkul et al. (2010b) observed the photodegradation of PBAT film in Costa Rica soils, with the formation of free radicals. PBAT films with greater aromatic content present a greater degree of crosslinking during irradiation. Stloukal et al., (2012) found that the mineralization in composting was not influenced by the level of crosslinking or photooxidation. However, Kijchavengkul et al. (2008) discovered that the same effect caused a decrease in the biodegradation of PBAT films in composting. Even other factors such as moisture may interact with photodegradation, leading to unpredictable results (Nikolić et al., 2017). This and other factors, including nutrients and type of soil could be considered to enhance the quality of the model. Another aspect that should be addressed is the characteristics of the polymers being tested. Phase transition temperatures (glass transition or melting temperature) of the polymers in this range (20-37 °C) can change the biodegradation process. For example, for PLA, above the glass transition temperature, the biodegradation is improved, because the chains in the amorphous regions become flexible (Iovino et al., 2008). These types of questions need to be taken into consideration when testing other polymers.

5.5 CONCLUSION

The key parameter for the biodegradation efficiency of this bilayer film in soil was the temperature, within the range between 37 and 45 °C. In this case, to accelerate the biodegradation in soil, a value of 37 °C was found as the best, since it requires less energy, than the higher temperatures tested. The range of MHC (60 – 100 %) tested, resulted in similar outcomes indicating that any value within this range can be used to properly test biodegradation of plastics. The addition of nitrogen didn't influence biodegradation, which indicates that the soil contained the nitrogen necessary and an adequate C:N ratio (within the range recommended by the standards).

The model developed can be used as a reliable tool that allows the simulation of the biodegradation of these films when introduced into similar soils and in the range of the parameters tested. In further works,

this approach can be used to test the biodegradation of this material in other environments (fresh and marine water) and test different relevant polymer blends or composites.

5.6 REFERENCES

ASTM D1293-18. (2018). Standard Test Methods for pH of Water. *ASTM International*.

ASTM D425-17. (2017). Test Method for Centrifuge Moisture Equivalent of Soils. *ASTM International*.

ASTM D5988-18. (2018). Standard Test Method for Determining Aerobic Biodegradation in Soil of Plastic Materials. *ASTM International.*

Beber, V. C., de Barros, S., Banea, M. D., Brede, M., de Carvalho, L. H., Hoffmann, R., ... Wellen, R. M. R. (2018). Effect of Babassu natural filler on PBAT/PHB biodegradable blends: An investigation of thermal, mechanical, and morphological behavior. *Materials*, 11(5). Retrieved from https://doi.org/10.3390/ma11050820

Briassoulis, D, & Mistriotis, A. (2018). Key parameters in testing biodegradation of bio-based materials in soil. *Chemosphere*, 207, 18–26. Retrieved from https://doi.org/10.1016/j.chemosphere.2018.05.024

Briassoulis, Demetres, Mistriotis, A., Mortier, N., & Tosin, M. (2020). A horizontal test method for biodegradation in soil of bio-based and conventional plastics and lubricants. *Journal of Cleaner Production*, 242, 118392. Retrieved from https://doi.org/https://doi.org/10.1016/j.jclepro.2019.118392

Camani, P. H., Souza, A. G., Barbosa, R. F. S., Zanini, N. C., Mulinari, D. R., & Rosa, D. S. (2021). Comprehensive insight into surfactant modified-PBAT physico-chemical and biodegradability properties. *Chemosphere*, 269, 128708. Retrieved from https://doi.org/10.1016/j.chemosphere.2020.128708

Chivrac, F., Kadlecová, Z., Pollet, E., & Avérous, L. (2006). Aromatic copolyester-based nano-biocomposites: Elaboration, structural characterization and properties. *Journal of Polymers and the Environment*, 14(4), 393–401. Retrieved from https://doi.org/10.1007/s10924-006-0033-4

Frey, S. D., Elliott, E. T., Paustian, K., & Peterson, G. A. (2000). Fungal translocation as a mechanism for soil nitrogen inputs to surface residue decomposition in a no-tillage agroecosystem. *Soil Biology and Biochemistry*, 32(5), 689–698. Retrieved from https://doi.org/https://doi.org/10.1016/S0038-0717(99)00205-9

Fu, Y., Wu, G., Bian, X., Zeng, J., & Weng, Y. (2020). Biodegradation Behavior of Poly(Butylene Adipate-Co-Terephthalate) (PBAT), Poly(Lactic Acid) (PLA), and Their Blend in Freshwater with Sediment. *Molecules*, 25(17). Retrieved from https://doi.org/10.3390/molecules25173946

Güsewell, S., & Gessner, M. O. (2009). N : P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. *Functional Ecology*, 23(1), 211–219. Retrieved from https://doi.org/https://doi.org/10.1111/j.1365-2435.2008.01478.x

Han, Y., Teng, Y., Wang, X., Ren, W., Wang, X., Luo, Y., ... Christie, P. (2021). Soil Type Driven Change in Microbial Community Affects Poly(butylene adipate- co-terephthalate) Degradation Potential. *Environmental Science and Technology*, 55(8), 4648–4657. Retrieved from https://doi.org/10.1021/acs.est.0c04850

Hodge, A., Robinson, D., & Fitter, A. (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science*, 5(7), 304–308. Retrieved from https://doi.org/https://doi.org/10.1016/S1360-1385(00)01656-3

Hoshino, A., Sawada, H., Yokota, M., Tsuji, M., Fukuda, K., & Kimura, M. (2001). Influence of weather conditions and soil properties on degradation of biodegradable plastics in soil. *Soil Science and Plant Nutrition*, 47(1), 35–43. Retrieved from https://doi.org/10.1080/00380768.2001.10408366

Iovino, R., Zullo, R., Rao, M. A., Cassar, L., & Gianfreda, L. (2008). Biodegradation of poly(lactic acid)/starch/coir biocomposites under controlled composting conditions. *Polymer Degradation and Stability*, 93(1), 147–157. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2007.10.011

Joyyi, L., Ahmad Thirmizir, M. Z., Salim, M. S., Han, L., Murugan, P., Kasuya, K., … Sudesh, K. (2017). Composite properties and biodegradation of biologically recovered P(3HB-co-3HHx) reinforced with short kenaf fibers. *Polymer Degradation and Stability*, 137, 100–108. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.004

Kijchavengkul, T., Auras, R., Rubino, M., Ngouajio, M., & Fernandez, R. T. (2008). Assessment of aliphatic-aromatic copolyester biodegradable mulch films. Part II: Laboratory simulated conditions. *Chemosphere*, 71(9), 1607–1616. Retrieved from https://doi.org/10.1016/j.chemosphere.2008.01.037

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2010a). Biodegradation and hydrolysis rate of aliphatic aromatic polyester. *Polymer Degradation and Stability*, 95(12), 2641–2647. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.07.018

Kijchavengkul, T., Auras, R., Rubino, M., Alvarado, E., Camacho Montero, J. R., & Rosales, J. M. (2010b). Atmospheric and soil degradation of aliphatic-aromatic polyester films. *Polymer Degradation and Stability*, 95(2), 99–107. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.11.048

Lim, S.-P., Gan, S.-N., & Tan, I. K. P. (2005). Degradation of medium-chain-length polyhydroxyalkanoates in tropical forest and mangrove soils. *Applied Biochemistry and Biotechnology*, 126(1), 23–33. Retrieved from https://doi.org/10.1007/s12010-005-0003-7

Matthies, M., & Beulke, S. (2017). Considerations of temperature in the context of the persistence classification in the EU. *Environmental Sciences Europe*, 29(1), 15. Retrieved from https://doi.org/10.1186/s12302-017-0113-1

Mergaert, J., Webb, A., Anderson, C., Wouters, A., & Swings, J. (1993). Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. *Applied and Environmental Microbiology*, 59(10), 3233–3238. Retrieved from https://doi.org/10.1128/aem.59.10.3233-3238.1993

Nikolić, M. A. L., Gauthier, E., Colwell, J. M., Halley, P., Bottle, S. E., Laycock, B., & Truss, R. (2017). The challenges in lifetime prediction of oxodegradable polyolefin and biodegradable polymer films. *Polymer Degradation and Stability*, 145, 102–119. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2017.07.018

Nishide, H., Toyota, K., & Kimura, M. (1999). Effects of soil temperature and anaerobiosis on degradation of biodegradable plastics in soil and their degrading microorganisms. *Soil Science and Plant Nutrition*, 45(4), 963–972. Retrieved from https://doi.org/10.1080/00380768.1999.10414346

Pischedda, A., Tosin, M., & Degli-Innocenti, F. (2019). Biodegradation of plastics in soil: The effect of temperature. *Polymer Degradation and Stability*, 170, 109017. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2019.109017

Purahong, W., Wahdan, S. F. M., Heinz, D., Jariyavidyanont, K., Sungkapreecha, C., Tanunchai, B., … Buscot, F. (2021). Back to the Future: Decomposability of a Biobased and Biodegradable Plastic in Field Soil Environments and Its Microbiome under Ambient and Future Climates. *Environmental Science & Technology*, 55(18), 12337–12351. Retrieved from https://doi.org/10.1021/acs.est.1c02695

Qi, R., Jones, D. L., Liu, Q., Liu, Q., Li, Z., & Yan, C. (2021). Field test on the biodegradation of poly(butylene adipate-co-terephthalate) based mulch films in soil. *Polymer Testing*, 93, 107009. Retrieved from https://doi.org/10.1016/j.polymertesting.2020.107009

Rodrigues, M., & Iemma, A. (2014). *Experimental Design and Process Optimization CRC Press, 2014*.

Roosen, M., Mys, N., Kusenberg, M., Billen, P., Dumoulin, A., Dewulf, J., ... De Meester, S. (2020). Detailed Analysis of the Composition of Selected Plastic Packaging Waste Products and Its Implications for Mechanical and Thermochemical Recycling. *Environmental Science & Technology*, 54(20), 13282–13293. Retrieved from https://doi.org/10.1021/acs.est.0c03371

Rosa, D. S., Lotto, N. T., Lopes, D. R., & Guedes, C. G. F. (2004). The use of roughness for evaluating the biodegradation of poly-β-(hydroxybutyrate) and poly-β-(hydroxybutyrate-co-β-valerate). *Polymer Testing*, 23(1), 3–8. Retrieved from https://doi.org/10.1016/S0142-9418(03)00042-4

Sabapathy, P. C., Devaraj, S., Parthipan, A., & Kathirvel, P. (2019). Polyhydroxyalkanoate production from statistically optimized media using rice mill effluent as sustainable substrate with an analysis on the biopolymer's degradation potential. *International Journal of Biological Macromolecules*, 126, 977–986. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.01.003

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology*, 218(12), 1856–1866. Retrieved from https://doi.org/10.1242/jeb.118851

Schulte, P. M., Healy, T. M., & Fangue, N. A. (2011). Thermal Performance Curves, Phenotypic Plasticity, and the Time Scales of Temperature Exposure. *Integrative and Comparative Biology*, 51(5), 691–702. Retrieved from https://doi.org/10.1093/icb/icr097

Šerá, J., Serbruyns, L., De Wilde, B., & Koutný, M. (2020a). Accelerated biodegradation testing of slowly degradable polyesters in soil. *Polymer Degradation and Stability*, 171, 109031. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2019.109031

Šerá, J., Kadlečková, M., Fayyazbakhsh, A., Kučabová, V., & Koutný, M. (2020b). Occurrence and analysis of thermophilic poly(Butylene adipate-co-terephthalate)-degrading microorganisms in temperate zone soils. *International Journal of Molecular Sciences*, 21(21), 1–17. Retrieved from https://doi.org/10.3390/ijms21217857

Silva, P M, Prieto, C., Andrade, C. C. P., Lagarón, J. M., Pastrana, L. M., Coimbra, M. A., ... Cerqueira, M. A. (2022). Hydroxypropyl methylcellulose-based micro- and nanostructures for encapsulation of melanoidins: Effect of electrohydrodynamic processing variables on morphological and physicochemical properties. *International Journal of Biological Macromolecules*, 202, 453–467. Retrieved from https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.01.019

Silva, Pedro M, Martins, A. J., Fasolin, L. H., & Vicente, A. A. (2021). Modulation and Characterization of Wax-Based Olive Oil Organogels in View of Their Application in the Food Industry. *Gels*, 7(1), 1–18. Retrieved from https://doi.org/10.3390/gels7010012

SM 2540 G. (1998). Total, Fixed, and Volatile Solids in Solid and Semisolid Samples. *Standard Methods for the Examination of Water and Wastewater, APHA-AWWA-WPCF. (20th ed.)*

Stloukal, P., Verney, V., Commereuc, S., Rychly, J., Matisova-Rychlá, L., Pis, V., & Koutny, M. (2012). Assessment of the interrelation between photooxidation and biodegradation of selected polyesters after artificial weathering. *Chemosphere*, 88(10), 1214–1219. Retrieved from https://doi.org/https://doi.org/10.1016/j.chemosphere.2012.03.072

Tarrahi, R., Fathi, Z., Seydibeyoğlu, M. Ö., Doustkhah, E., & Khataee, A. (2020). Polyhydroxyalkanoates (PHA): From production to nanoarchitecture. *International Journal of Biological Macromolecules*, 146, 596–619. Retrieved from https://doi.org/10.1016/j.ijbiomac.2019.12.181

Teixeira, P. F., Covas, J. A., Suarez, M. J., Angulo, I., & Hilliou, L. (2020). Film Blowing of PHB-Based

Systems for Home Compostable Food Packaging. *International Polymer Processing*, 35(5), 440–447. Retrieved from https://doi.org/10.3139/217.3985

Zhou, J., Gui, H., Banfield, C. C., Wen, Y., Zang, H., Dippold, M. A., ... Jones, D. L. (2021). The microplastisphere: Biodegradable microplastics addition alters soil microbial community structure and function. *Soil Biology and Biochemistry*, 156(February), 108211. Retrieved from https://doi.org/10.1016/j.soilbio.2021.108211

CHAPTER 6.

BIODEGRADATION OF PBAT AND PHB/PBAT BY *ASPERGILLUS* AND *PURPUREOCILLIUM* ISOLATES

6.1 INTRODUCTION

PBAT is produced from adipic acid, 1,4-butanediol and terephthalic acid, and has analogous mechanical properties to conventional low-density polyethylene (LDPE) (Yamamoto et al., 2005). It can be degraded at thermophilic temperatures in composting settings (Kijchavengkul et al., 2010). However, in other environments such as soil, the degradation is considerably slower or inexistent (Han et al., 2021). The slower biodegradation rate when compared with PHAs polymers, is related to the limiting number of microorganisms capable of degrading PBAT in the environment, and the difficult action of the enzymes on terephthalic acid ester bonds (Soulenthone et al., 2020). The degradation of PBAT is well studied with compost isolates, namely with *Thermobifida fusca* at high temperatures (55 °C), which can achieve almost complete degradation in 22 days (Witt et al., 2001). The *Thermobifida fusca* DSM 43793 was found to act as an initial degrader, converting PBAT into lower molecular mass products, which were further degraded by other microorganisms, as *Thermobifida fusca* is unable to metabolize those degradation products (Kleeberg et al., 2005; Witt et al., 2001). Under mesophilic temperatures only very few isolated aerobic microorganisms, such as *Bacillus subtilis* ATCC 21332, *Bacillus pumilus* NKCM 3201, *Cryptococcus* sp. MTCC 5455, *Isaria* sp. NKCM 1712 and *Leptothrix* sp. TB-71 demonstrated the capacity to degrade PBAT (Aarthy et al., 2018; Kasuya et al., 2009; Muroi et al., 2017; Nakajima-Kambe et al., 2009a; Trinh Tan et al., 2008). Generally, more than 50 days are necessary to achieve partial degradation (i.e., 43 %) of PBAT under aerobic mesophilic conditions (Kasuya et al., 2009).

The degradation is even slower in anaerobic environments and known microorganisms are rare. *Hungatella hathewayi* DSM 13479 and *Pelosinus fermentans* are examples of anaerobes that can biodegrade PBAT, although more than 14 days are necessary to biodegrade about 5 % (Biundo et al., 2016; Perz et al., 2016a).

Cocultures of *Pseudomonas mendocina* and *Actinomucor elegans* were able to degrade 18.95 % in weight of PLA/PBAT films in 5 days, under aerobic and mesophilic conditions. This coculture was far more efficient than the pure cultures alone since in monocultures *Pseudomonas mendocina* and *Actinomucor elegans* could only degrade 12.94 % and 9.27 % (in weight), respectively (Jia et al., 2021).

In this chapter, two fungi isolated from soil (Chapter 4) were tested for their potential to biodegrade films of PBAT and PHB/PBAT. Biodegradation tests were performed in mono and coculture, in liquid media and under mesophilic conditions. The potential enzymes involved in PBAT biodegradation were investigated as well.

6.2 MATERIALS AND METHODS

6.2.1 Microorganisms: selection, storage and maintenance

The two fungi used in this study were isolated in the work described in Chapter 4. They both grow under aerobic and mesophilic conditions (30 °C). The isolates were assigned to *Aspergillus pseudoflectus* (Isolate 7) and *Purpureocillium lilacinum* (Isolate 9), being closely related to *Aspergillus pseudoflectus* strain isolate CCMG111 (99.83 % similarity at the ITS level) and *Purpureocillium lilacinum* clone SF_357 (100 % similarity at the ITS level) (Table 4.8).

Before the assays, spores stock solutions were prepared as described by Simões et al. (2015). Briefly, spores from isolate 7 and isolate 9 were collected from ten-day-old pure cultures grown in Potato Dextrose Agar (VWR Chemicals) at 30 °C, by flooding the surface of the agar plates with 2 mL of TWS solution (0.85 % NaCl plus 0.05 % Tween 80) and shaking gently. The suspension was then homogenized by vortexing and used for large-scale production of spores. The final spore suspension was homogenized by vortexing before quantification using a Neubauer count chamber. Some aliquots of spore suspension were cryopreserved at -80 °C in 20 % glycerol, to allow using the same spore suspension in all assays. The spores were pre-germinated, to stimulate their metabolic state. The germinated spores were prepared by inoculating the aliquots in nutrient broth (VWR Chemicals) for 18 h (time necessary for the germination of > 95 % of the spores). Before use, these suspensions were always washed twice to eliminate the carbon sources, by centrifugation for 30 s, and resuspended in phosphate-buffered saline solution. This final suspension was also homogenized by vortexing before quantification using a Neubauer count chamber, to control the concentration of spores used in the assays.

6.2.2 Polymers films

The plastic used was the polyhydroxybutyrate (PHB)/PBAT 55 %/45 % in weight bilayer films described in section 3.2.2.

6.2.3 Clear zone methodology

A mineral salts medium with only PBAT as carbon source was prepared accordingly to 4.2.4 Agar plate **containing plastic**. The two isolates were tested in this medium for the formation of clear zones, at different temperatures, more precisely between 20 °C and 37 °C.

6.2.4 Biodegradation in liquid media

Erlenmeyer shake flasks (125 mL) capped with foam plugs were prepared for the screening tests. A mineral salt medium with no carbon source was prepared according to section 3.2.4, except that neither agar nor dispersed polymers were added. The shake flasks with 10 mL of media were sterilized with saturated steam at 121 °C in an autoclave for 15 min. In each flask, one PHB/PBAT film (2 x 2 cm) sterilized (by soaking it in ethanol, followed by rinsing it with sterile water) was added. After inoculation, the shake flasks were incubated at 28 °C and 250 rpm in a rotary shaker for 14 days. the residual films were recovered, rinsed with distilled water, and dried to a constant weight. The films were weighted before sterilization. The weight loss of the film was determined by subtracting its weight after degradation from its initial weight. The inoculum for each shake flask was $2x10^{\wedge 4}$/mL, and for the coculture equal concentration of each isolate was inoculated. A negative control experiment (abiotic experiment) was performed without adding any isolate. Because the film was composed of 2 polymers, and PBAT is normally more difficult to degrade than PHB, another set of tests was conducted to evaluate the PBAT film (2 x 2 cm) biodegradation solely.

6.2.5 Biochemical oxygen demand (BOD) tests

Measurements of oxygen (O_2) consumption were carried out using a closed system method with air as the initial atmosphere. Briefly, the experiments were carried out in glass bottles of 120 mL and used as reactors to evaluate the biodegradation of the films. 60 mL of the same carbon-free medium was placed in each bottle along with PBAT films (1 cm x 4 cm). After autoclaving, the bottles were prepared in triplicate, and the tested conditions were, inoculation with isolate 7 or isolate 9, with both organisms in coculture, and without microorganisms (control). In each bottle, 33 mg of fungi were added. Nutrient broth (VWR Chemicals) was used as a positive control for each fungus (duplicates). The systems were closed and stored at 27 °C under agitation (200 rpm). The concentrations of O_2 inside the containers were measured by drawing gas samples with a 500 µL syringe suitable for gas chromatography through a silicone septum fitted in the bottle lids. The O_2 consumption was measured until it was kept constant. The O_2 content was analysed through a gas chromatograph (Bruker Scion 456, Markham, ON, Canada), equipped with a thermal conductivity detector (TCD) at 130 °C, and a Molsieve column and Argon (30 mL/min) as the carrier gas was used to separate O_2 (Ballesteros et al., 2022). Normal air was used as a standard sample for calibration.

6.2.6 Identification of enzymes potentially involved in PBAT biodegradation

To identify the enzymes responsible for PBAT degradation by the isolates, a literature search was performed to collect information on enzymes already described as involved in the degradation of PBAT. Such information was compiled in Table 6.1. Those 26 enzymes were used as reference to search for potential PBAT degrading enzymes that could be expressed by the isolates.

Table 6.1 Microorganisms or/and enzymes identified from the literature as capable of degrading PBAT

Enzyme	Microorganism	NCBI ID	GenBank/ UniProt/MGnify	EC number	PDB entry	References
Polyesterase	*Pseudomonas pseudoalcaligenes*	WP_003460012	W6R2Y2	EC 3.1.1.74		(Wallace et al., 2017)
Hydrolase	*Rhodococcus fascians* NKCM251	BCL64964	A0A7I8E2Z4			(Soulenthone et al., 2021)
Cutinase	*Thermobifida cellulosilytica*	ADV92526	ADV92526.1	EC 3.1.1.74	5LUI	(Perz et al., 2016a)
		ADV92527			5LUI	
Cutinase	*Thermobifida alba*	BAI99230	BAI99230	EC 3.1.1.1.74		(Thumarat et al., 2015)
Cutinase	*Thermobifida alba*	BAK48590	F7IX06	EC 3.1.1.1.74		(Thumarat et al., 2012)
Hydrolase	*Pelosinus fermentans* DSM 17108	AIX10936	EIW29778.1			(Biundo et al., 2016)
Esterase	*Clostridium botulinum*	AKZ20828	AKZ20828.1			(Perz et al., 2016b)
		AKZ20829	AKZ20829.1			
Hydrolase	*Bacillus pumilus*	BAV72205	A0A1E1FNX8			(Muroi et al., 2017)
Polyesterase	*Saccharomonospora viridis*	WP_015787089 /BAO42836				(Kawai et al., 2014)
Carboxylesterase	uncultured bacterium	AOR05748				(Müller et al., 2017)
Carboxylesterase	*uncultured bacterium*	AOR05749				
Carboxylesterase	*uncultured bacterium*	AOR05750				
Carboxylesterase	*uncultured bacterium*	AOR05751				
Carboxylesterase	*uncultured bacterium*	AOR05752				
Carboxylesterase	*uncultured bacterium*	AOR05753				

Cutinase	*Humicola insolens*	QAY29138	A0A075B5G4	EC 3.1.1.74	4OYL and others	(Perz et al., 2016a)
Cutinase	*Fusarium solani*	AAA33334		Novozym® 51032		(Zumstein et al., 2017)
Lipase	*Rhizopus oryzae*	Organism (AB721967)/1TIC		sigma 80612/62305		(Zumstein et al., 2017)
Esterase	*Leptothrix sp.* strain TB-71	Organism (AB458235)				(Nakajima-Kambe et al., 2009; Shah et al., 2014)
Esterase	*Hungatella hathewayi* DSM 13479	ALS54749				(Perz et al., 2016c)
Hydrolase	*Thermobifida fusca* DSM 43793	CAH17554				(Kleeberg et al., 2005; Müller et al., 2005)
		CAH17553				
Cutinase	*Paraphoma* sp. B47-9	BAN51853				(Suzuki et al., 2014)
Cutinase	*Saitozyma flava (Cryptococcus flavus)*	BAT32793				(Watanabe et al., 2015)

The genomes of the isolates 7 and 9 are not sequenced and for this reason, the search was done against the closest relatives whose genomes are available in public databases, which were tax ID 33203 (*Purpureocillium lilacinum*), and tax ID 5052 (*Aspergillus* genus), for isolate 9 and 7, respectively. The search for enzymes homologous to those known to degrade PBAT in the selected genomes was performed with M-party, a bioinformatics tool developed in-house and available at Bioconda (https://bioconda.github.io/recipes/m-party/README.html). Briefly, this tool builds Hidden Markov Models (HMMs) based on reference proteins, extends the reference protein datasets by including other protein sequences belonging to the same protein family, and finds homologous proteins in FASTA protein datasets. The extension of the reference database has the objective of increasing the number of reference enzymes and this way increasing the probability of finding similar enzymes in the proteomes of the microorganisms. The cutinase family (EC 3.1.1.74) was used in the extension and the information was retrieved from the KEGG database.

6.2.7 Statistical analysis

Biodegradation trials were performed in Erlenmeyer flasks, and the BOD assays were analysed using the OriginPRO 2019b statistical program by one-way analysis of variance (ANOVA) followed by the Fisher LSD test, and statistical significance was accepted as $p < 0.05$ (95 % significance).

6.3 RESULTS

6.3.1 Evaluation of clear zones formation

The clear zone assays demonstrated that both isolates are capable of degrading PBAT at temperatures between 20 and 30 °C, although faster clear zones were formed at 30 °C. At 37 °C no clear zones were formed as indicated in Table 6.2, suggesting that these microorganisms have no activity on PBAT at this temperature. Soulenthone et al. (2020) indicated similar outcomes from *Rhodococus fascians* NBRC 100625 and strain NKCM 2511, with these microorganisms having an optimum temperature range from 25 °C to 30 °C, but not forming clear zones at and above 37 °C, in PBAT agar plates.

Table 6.2 Effect of temperature on the clear zone formation of the isolates in PBAT emulsified agar plates after 7 days. -:no clearing zone formation, +: radius of clear zone smaller than 1 cm, ++: radius of clear zone between 1 and 3 cm. +++: radius of clear zone superior to 3 cm. All experiments were performed in triplicate

Temperature (°C)	Isolate 7 (*Aspergillus pseudoflectus* sp.)	Isolate 9 (Purpureocillium lilacinum sp.)
	Clear zone formation	
20	++	++
25	+++	+++
30	+++	+++
37	-	-

6.3.2 Polymers degradation in liquid media

The two isolates could degrade the PHB/PBAT film, although isolate 9 was slightly more efficient, degrading more than 43 %, while isolate 9 degraded about 41 % (Table 6.3). The biodegradation rate for isolate 7 was 2.9 %/day while for isolate 9 it was 3.1 %/day. The coculture formed by the two isolates together was slightly less efficient (2.9 %/day) than isolate 9 alone and was as efficient as isolate 7. The two microorganisms performed similarly and the coculture was less efficient maybe due to competition for the surface area. Some abiotic degradation occurred in the control assay (Table 6.3), nevertheless, the biodegradation in all conditions tested was significantly different in relation to the control assay, but not between each other.

Table 6.3 Degradation of PHB/PBAT and PBAT polymer films after 14 days of incubation at 30 °C. All experiments were performed in triplicate

Microorganism (Isolate/Genus)	Average weight loss (%)	
	PHB/PBAT film	PBAT film
Isolate *7 (Aspergillus* sp.*)*	40.7 ± 5.3[a]	2.0 ± 0.2[3]
Isolate *9 (Purpureocillium sp.)*	43.4 ± 4.3[a]	2.4 ± 0.4[2]
Coculture (Isolate 7 and 9)	40.6 ± 0.6[a]	2.6 ± 0.3[2]
Control	3.8 ± 0.5[b]	0.4 ± 0.3[1]

For each column, different letters or numbers correspond to statistically significant differences ($p < 0.05$).

The results show the lower biodegradation of PBAT at a temperature of 30 °C (Table 6.3). All the conditions were significantly different in relation to the control assay. PBAT biodegradation by isolate 7 was also significantly different from isolate 9 and the co-culture. Isolate 9 showed a higher biodegradation rate (0.17 %/day) when compared to isolate 7 (0.14 %/day), and the highest weight loss was obtained by the coculture (0.19 %/day). The results using only PBAT films confirmed that the weight loss in the first set of trials (Table 6.3) was mainly due to the action of the fungi on the PHB layer because the weight loss with PBAT films was substantially lower. Remarkably, both isolates can biodegrade the two polymers, but when both are available, preference is given to PHB as the carbon source.

6.3.3 BOD trial

The biological oxygen demand experiment (Table 6.4) demonstrated that most of the oxygen consumption in the bottles occurred in the first 60 days for all conditions. From thereafter, the consumption was slower. Isolate 9 was more efficient reaching 41.0 % biodegradation while the isolated 7 reached 31.2 %. The co-culture achieved 28.8 % biodegradation, and even in the negative control, some degradation was also detected (13.8 %), which corresponds to abiotic biodegradation. The colonization of the PBAT surface by isolate 7, which is less efficient in PBAT biodegradation, may explain the lowest mineralization obtained by the coculture. The colonization can be seen in Figure S1 - Appendix from Chapter 6. All the conditions were significantly different in relation to the control after 287 days and Isolate 9 achieved the highest biodegradation that was also significantly different from the other conditions

Table 6.4 Biodegradation of PBAT films by isolate 7, isolate 9 and the coculture, accessed by BOD tests. All experiments were performed in triplicate

Microorganism (Isolate/Genus)	PBAT (mg)	O_2 Consumed ($mmol.L^{-1}$)	O_2 Consumed (mmol)	Mineralization (%)	Mineralization without control (%	O_2 Consumed ($mmol.L^{-1}$)	O_2 Consumed (mmol)	Mineralization (%)	Mineralization without control (%)
		(After 35 days)				(After 287 days)			
Isolate 7 *(Aspergillus)*	16	2.7	0.2	17.1	5.3	4.9	0.3	30.2[b]	17.4
Isolate 9 (*Purpureocillium*)	16	4.2	0.3	27.0 (0)	15.2	6.1	0.4	41.2[c]	28.4
Coculture	16	2.6	0.2	16.6	4.8	4.3	0.3	27.4[b]	14.6
Control	16	1.9	0.1	11.8		2.2	0.1	12.8[a]	-
Isolate *7 (*Control*)*	-	3.1	0.2	-		2.1	0.1	-	-
Isolate 9 (Control)	-	4.7	0.3	-		3.4	0.2	-	-

Letters a, b and c correspond to statistically significant differences ($p < 0.05$)

6.3.4 Identification of enzymes potentially involved in PBAT biodegradation

By using the M-party bioinformatics tool, a total of 420 HMMs were obtained. Twelve different proteins could be detected in the genome of *Purpureocillium lilacinum* which are closely related to enzymes previously known to be involved in PBAT biodegradation. These proteins were found by 50 different HMMs, meaning that some proteins were detected more than once (Table 6.5). Although a positive match was obtained for 12 proteins, the function of some of those was not the same as the enzymes present in the initial dataset (Table 6.1), corresponding to false positives. For instance, 6 of the positive hits were annotated as amino acid transporter transmembrane domain-containing protein, ubiquinone biosynthesis protein COQ4, mitochondrial (Coenzyme Q biosynthesis protein) and 4 as beta-lactamases (data not shown). Therefore, only the remaining 6 proteins were considered as a valid result and were identified as cutinases (4 enzymes) and acetylxylan esterases (2 enzymes). These results make sense since these two types of enzymes were present in the reference dataset, as can be seen in Table 6.1. Because isolate 9 is very closely related to *Purpureocillium lilacinum* most probably similar enzymes can be also expressed by isolate 9 during the biodegradation of PBAT.

Table 6.5 Enzymes from *Purpureocillium lilacinum* (tax ID 33203) identified with the models as potential PBAT degrading enzymes

Purpureocillium lilacinum				
Uniprot ID	**Protein names**	**Gene Names**	**Microorganism**	**Number of HMMs that identify the protein**
A0A179GYZ5	Cutinase	VFPBJ_05098	*Purpureocillium lilacinum*	7
A0A179HT03	Cutinase	VFPFJ_04300		6
A0A2U3DZK7	Cutinase	PCL_02616		6
A0A179GM63	Cutinase	PCL_04231 VFPBJ_06338 VFPFJ_11216		40
A0A2U3DU00	Acetylxylan esterase	PCL_06909		1
A0A179H4M9	Acetylxylan esterase	VFPFJ_07534		1

Regarding the search on microorganisms assigned to the *Aspergillus* genus, 84 HMMs could identify 669 proteins, and similarly to what was obtained for *Purpureocillium lilacinum*, only 440 were closely related

to the enzymes present in the reference dataset. These included 8 acyltransferases, 405 cutinases, 13 esterases and 14 transesterase. The genes coding to these enzymes were found in the genomes of 79 different microorganisms belonging to the *Aspergillus* genus. The IDs (UniProt) of the enzymes are indicated in Table S1 – Appendix 6. Isolate 7 probably contains genes coding for proteins with these functions, that were all, or some of them, expressed during PBAT biodegradation.

Both *Purpureocillium lilacinum* and microorganisms assigned to the *Aspergillus* genus show high potential for PBAT biodegradation since they have several genes in their genomes coding for enzymes necessary for PBAT biodegradation. However, this was the first study describing their PBAT degrading activity.

6.4 DISCUSSION

Temperature clearly influenced the clear zone formation (Table 6.2), indicating that both fungi are mesophilic degraders with ideal growth between 25 °C and 30 °C. In all incubations, it was possible to see a fast weight loss of the PHB/PBAT films (Table 6.3), however with only PBAT films the weight loss was inferior. In studies with *Isaria fumosorosea* strain NKCM1712 it was shown that PBAT was degraded at the highest rate when PBAT was the only carbon source (Kasuya et al., 2009). With olive oil and tributyrin (lipids), and with the PBAT individual components the rate of PBAT film degradation was the same, indicating that these compounds do not suppress the expression of the PBAT enzyme. Nonetheless, with glucose and fructose, the fungus did not present substantial levels of hydrolytic action, which suggests that the PBAT hydrolytic action is regulated by catabolite repression in the presence of easily accessible carbon sources (Kasuya et al., 2009). With some types of carbon sources (starch, tryptone, and peptone) in high concentrations, the degradation of PBAT by the thermophilic actinomycete *Thermobifida fusc*a in agar plates was inhibited (Kleeberg et al., 1998). A bacterium from the genus *Rhodococus* demonstrated high PBAT degrading activity in LB media and also with olive oil, succinic acid, and 6-Hydroxyhexanoic acid as carbon sources (Soulenthone et al., 2020). However, with fructose, citric acid, adipic acid, ethylene glycol, 16-hydroxyhexadecanoic acid, and poly(ε-caprolactone) the activity was considered low. This type of phenomenon may explain the results and the PHB preference found here, being the PHB more easily available, or the expression/activity of the PBAT enzymes were inhibited.

The biodegradation in the BOD assay for all conditions was initially faster and slowed down considerably with the extension of the assay. Is possible that the fungi studied in this work are capable of degrading one or more specific bonds of the PBAT polymer and growing using one or more products as subtract. However, is also possible that these fungi cannot metabolize all the carbon present in the PBAT matrix.

For example, *Bacillus subtilis* ATCC 21332 was capable of easily degrading three small esters, namely tributyrin, dibutyl adipate and dibutyl terephthalate and also the aliphatic polyester, polybutylene adipate (Trinh Tan et al., 2008). Nevertheless, it was not capable of degrading the aromatic group polybutylene terephthalate. Other PBAT-degrading microorganism, namely *Leptothrix* sp. TB-71 used some adipic acid but not 1,4-butanediol (Nakajima-Kambe et al., 2009b). The bacteria *Bacillus pumilus* NKCM 3201 and *Thermobifida fusca* DSM 43793 did not use any PBAT constituents as subtracts to grow (Kleeberg et al., 2005; Muroi et al., 2017). In contrast, a *Rhodococcus* bacteria was capable of degrading PBAT and its constituents although at different rates, 55 % for 1,4-butanediol, 5 % for adipic acid, 7 % for terephthalic acid, and 7 % for PBAT (Soulenthone et al., 2020). PBAT is composed of an aromatic and an aliphatic group. The existence of the aromatic group in the polyester chain increases the resistance due to the lower hydrolytic degradation rate when compared with polyester with just aliphatic groups such as polylactic acid and polyglycolic acid (Kijchavengkul et al., 2010). This particular group, decreases the chain flexibility, creating fewer susceptible bonds, and originating a steric interference effect to the access of the more vulnerable ester bonds (Van Krevelen and Te Nijenhuis, 2009). For example, a lipase from *Pseudomonas* sp. had more difficulty cleaving esters near terephthalic acid in PBAT structure when compared to esters containing only aliphatic sequences (Marten et al., 2005).

Other factors such as temperature may influence the rates of enzymatic degradation of PBAT since it was found that a lipase from a *Pseudomonas* bacterium was clearly higher at 50 °C than at 37 °C, indicating the authors that this could be due to the physical form of PBAT at medium and high temperatures (Marten et al., 2005). It was noticed in the BOD assay some abiotic degradation (12.8 % in 287 days). This result is expected, given that Soulenthone et al. (2020) indicated that the molecular mass of PBAT film slightly decreased (5 % in 30 days) after incubation at 25 °C for 30 days implying an abiotically slow bulk hydrolysis. However, Kasuya et al. (2009) indicated opposite outcomes with no weight loss in PBAT films incubated in autoclaved soil at 30 °C for 30 days. Since in this work, the temperature was slightly higher (or similar) and the exposure time was much longer, it is not surprising that some abiotic degradation was detected. The mass loss of PBAT is also normally higher under thermophilic conditions, which may be attributed primarily to the influence of abiotic hydrolysis (Abou-Zeid et al., 2004).

At high temperatures (above 50 °C), thermophilic actinomycetes are the most important microorganisms involved in degrading PBAT (Kleeberg et al., 2005). However, at mesophilic temperatures, fungi from the phylum *Ascomycota* were found to be the major group of organisms capable of degrading PBAT (Kasuya et al., 2009; Trinh Tan et al., 2008). Some works indicate that bacteria (*Rhodococuss fascians*) at mesophilic temperatures presented a five times lower growth rate than fungi suggesting that the primary

degrading species of PBAT are fungi (*Isaria fumosorosea*) (Kasuya et al., 2009; Soulenthone et al., 2020). Muroi et al., (2016) found that fungi, especially *Ascomycota* have a dominant role in PBAT biodegradation in soil in mesophilic situations. These evidences highlight the potential use of the 2 fungi studied here in PBAT degradation-related solutions since they are likely to be more successful than bacteria.

The two microorganisms identified as PBAT degraders in this work are not taxonomically related to those described in the literature (Table 6.1), so this discovery increases the knowledge on the existing PBAT degraders. Several enzyme matches were found in the genome of closely related microorganisms, nevertheless, the two isolates don't have the genome sequenced, and the isolates may contain other, and even more effective enzymes than the already identified as PBAT degraders. The enzymes already identified with PBAT degrading activity have different preferences, such as for esters bonds with short acyl chains, or cleaving preferably adipic acid with 1,4 butanediol ester bonds than 1,4 butanediol with terephthalic acid ester bonds or even being capable of completely hydrolyse PBAT (Perz et al., 2016b; Suzuki et al., 2014; Zumstein et al., 2017). Trinh Tan et al. (2008) indicated an exo-mechanism for *Bacillus subtilis* enzymes since they degraded especially the lower molecular weight units. However, for polybutylene adipate, *Penicillium pinophilum* presented an endo mechanism with no accumulation of minor oligomeric compounds during the degradation of the polymer chains (Trinh Tan et al., 2008). To identify the enzymes of the isolates identified here, responsible for the degradation, further work needs to be conducted, such as transcriptomics assays during the biodegradation of the PBAT films. To enlighten the mechanism of action, in future works the enzymes should be isolated and tested to verify which ester bonds are cleaved, and what is the efficiency and the substrate specificity.

The degradation (2.0 + 0.1 wt %) of PBAT films after 21 days of exposure to *Bacillus subtilis* at 30 °C (Trinh Tan et al., 2008) was within the range found in this work. Nakajima-Kambe et al. (2009b) reported that *Leptothrix* sp. TB-71 degraded 1.4 ± 0.2 % in weight (30 °C) of PBAT in two weeks. This rate is considerably slower than the biodegradation of the two isolates studied in this chapter (Table 6.3); contrarily, *Isaria fumosorosea* NKCM 1712 presented a much higher rate, degrading 15 % in weight in 15 days of PBAT at 30 °C (Kasuya et al., 2009). All the analysis demonstrates the immense potential that resides in these two isolates, that can be explored.

Ideally, cocultures should increase the degradation rate in comparison with their applications as pure cultures. However, this is not always the case; for example, coculture of 1:1 *Thermobifida fusca*/ *Ideonella sakaiensis* had higher biodegradation efficiency of UV-treated PET pellets than *Ideonella sakaiensis* monoculture but lower than *Thermobifida fusca* monoculture (Lee et al., 2021). In this work, the coculture

1:1 isolate 7:isolate 9 in the BOD assay achieved a similar biodegradation efficiency compared to the isolate 7 monoculture but not compared to the isolate 9 monoculture, indicating the necessity for further optimization of the degradation conditions.

6.5 CONCLUSION

This is the first work to report the degradation of PBAT and PHB/PBAT films by monocultures of isolates assigned to *Purpureocillium lilacinum* and *Aspergillus pseudoflectus* and their respective coculture. The results indicate that the two microorganisms are aerobically mesophilic fungi capable of degrading solid PBAT. In all conditions the fungi could degrade the films, although the degradation was superior with PHB/PBAT films, indicating a clear preference for the PHB polymer. The monocultures and coculture were similarly efficient in the degradation of PBAT. The BOD revealed that the coculture did not achieve the best degradation result compared with the isolate 9 monoculture, indicating the necessity for optimization of the cultivation conditions. Considering all the results, in future works the enzymes of these microorganisms responsible for the PBAT degradation should be studied.

These microorganisms and their enzymes could be helpful in the management of environments with mixed plastic wastes, and for the monomer recycling process using for example enzymes with substrate specificity sequentially to make pure monomers from mixed plastic wastes. The understanding of the mechanism of degradation may also promote the industrial development of biodegradable plastics appropriate for enzymatic recycling, and the tailoring of enzymes with increased efficiency.

6.6 REFERENCES

Aarthy, M., Puhazhselvan, P., Aparna, R., George, A. S., Gowthaman, M. K., Ayyadurai, N., ... Kamini, N. R. (2018). Growth associated degradation of aliphatic-aromatic copolyesters by Cryptococcus sp. MTCC 5455. *Polymer Degradation and Stability*, 152, 20–28. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2018.03.021

Abou-Zeid, D. M., Müller, R. J., & Deckwer, W. D. (2004). Biodegradation of aliphatic homopolyesters and aliphatic-aromatic copolyesters by anaerobic microorganisms. *Biomacromolecules*, 5(5), 1687–1697. Retrieved from https://doi.org/10.1021/bm0499334

Ballesteros, L. F., Teixeira, J. A., & Cerqueira, M. A. (2022). Active Carboxymethyl Cellulose-Based Edible Coatings for the Extension of Fresh Goldenberries Shelf-Life, 10(8), 1-22. *Horticulturae*. Retrieved from https://doi.org/10.3390/horticulturae8100936

Biundo, A., Hromic, A., Pavkov-keller, T., Gruber, K., Quartinello, F., Haernvall, K., ... Guebitz, G. M. (2016). Characterization of a poly (butylene adipate- co -terephthalate) - hydrolyzing lipase from Pelosinus fermentans, *Applied Microbiology and Biotechnology,* 100, 1753–1764. Retrieved from

https://doi.org/10.1007/s00253-015-7031-1

Han, Y., Teng, Y., Wang, X., Ren, W., Wang, X., Luo, Y., ... Christie, P. (2021). Soil Type Driven Change in Microbial Community Affects Poly(butylene adipate- co-terephthalate) Degradation Potential. *Environmental Science and Technology*, 55(8), 4648–4657. Retrieved from https://doi.org/10.1021/acs.est.0c04850

Jia, H., Zhang, M., Weng, Y., & Li, C. (2021). Degradation of polylactic acid/polybutylene adipate-co-terephthalate by coculture of Pseudomonas mendocina and Actinomucor elegans. *Journal of Hazardous Materials*, 403(August), 123679. Retrieved from https://doi.org/10.1016/j.jhazmat.2020.123679

Kasuya, K. ichi, Ishii, N., Inoue, Y., Yazawa, K., Tagaya, T., Yotsumoto, T., ... Nagai, D. (2009). Characterization of a mesophilic aliphatic-aromatic copolyester-degrading fungus. *Polymer Degradation and Stability*, 94(8), 1190–1196. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.04.013

Kawai, F., Oda, M., Tamashiro, T., Waku, T., Tanaka, N., Yamamoto, M., ... Tanokura, M. (2014). A novel Ca^{2+}-activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from Saccharomonospora viridis AHK190. *Applied Microbiology and Biotechnology*, 98(24), 10053–10064. Retrieved from https://doi.org/10.1007/s00253-014-5860-y

Kijchavengkul, T., Auras, R., Rubino, M., Selke, S., Ngouajio, M., & Fernandez, R. T. (2010). Biodegradation and hydrolysis rate of aliphatic aromatic polyester. *Polymer Degradation and Stability*, 95(12), 2641–2647. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2010.07.018

Kleeberg, I, Welzel, K., VandenHeuvel, J., Müller, R.-J., & Deckwer, W.-D. (2005). Characterization of a New Extracellular Hydrolase from Thermobifida fusca Degrading Aliphatic–Aromatic Copolyesters. *Biomacromolecules*, 6(1), 262–270. Retrieved from https://doi.org/10.1021/bm049582t

Kleeberg, Ilona, Hetz, C., Kroppenstedt, R. M., Müller, R. J., & Deckwer, W. D. (1998). Biodegradation of aliphatic-aromatic copolyesters by Thermomonospora fusca and other thermophilic compost isolates. *Applied and Environmental Microbiology*, 64(5), 1731–1735. Retrieved from https://doi.org/10.1128/aem.64.5.1731-1735.1998

Lee, A., Mohd Sahari, S. S. B., & Liew, M. S. (2021). Feasibility study of a co-culture system for PET-degrading bacteria to increase biodegradation performance. *Bioremediation Journal*, 25(3), 197–203. Retrieved from https://doi.org/10.1080/10889868.2021.1900053

Marten, E., Müller, R. J., & Deckwer, W. D. (2005). Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic-aromatic copolyesters. *Polymer Degradation and Stability*, 88(3), 371–381. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2004.12.001

Müller, C. A., Perz, V., Provasnek, C., Quartinello, F., Guebitz, G. M., & Berg, G. (2017). Discovery of polyesterases from mossassociated microorganisms. *Applied and Environmental Microbiology*, 83(4). Retrieved from https://doi.org/10.1128/AEM.02641-16

Müller, R.-J., Schrader, H., Profe, J., Dresler, K., & Deckwer, W.-D. (2005). Enzymatic Degradation of Poly(ethylene terephthalate): Rapid Hydrolyse using a Hydrolase from T. fusca. *Macromolecular Rapid Communications*, 26(17), 1400–1405. Retrieved from https://doi.org/https://doi.org/10.1002/marc.200500410

Muroi, F., Tachibana, Y., Kobayashi, Y., Sakurai, T., & Kasuya, K. I. (2016). Influences of poly(butylene adipate-co-terephthalate) on soil microbiota and plant growth. *Polymer Degradation and Stability*, 129, 338–346. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2016.05.018

Muroi, F., Tachibana, Y., Soulenthone, P., Yamamoto, K., Mizuno, T., Sakurai, T., ... Kasuya, K. (2017). Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the aerobic mesophilic bacterium Bacillus pumilus. *Polymer Degradation and Stability*, 137, 11–22. Retrieved from

https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2017.01.006

Nakajima-Kambe, T., Ichihashi, F., Matsuzoe, R., Kato, S., & Shintani, N. (2009a). Degradation of aliphatic-aromatic copolyesters by bacteria that can degrade aliphatic polyesters. *Polymer Degradation and Stability*, 94(11), 1901–1905. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2009.08.006

Nakajima-Kambe, T., Toyoshima, K., Saito, C., Takaguchi, H., Akutsu-Shigeno, Y., Sato, M., … Uchiyama, H. (2009b). Rapid monomerization of poly(butylene succinate)-co-(butylene adipate) by Leptothrix sp. *Journal of Bioscience and Bioengineering*, 108(6), 513–516. Retrieved from https://doi.org/https://doi.org/10.1016/j.jbiosc.2009.05.018

Perz, V., Baumschlager, A., Bleymaier, K., Zitzenbacher, S., Hromic, A., Steinkellner, G., … Guebitz, G. M. (2016b). Hydrolysis of synthetic polyesters by Clostridium botulinum esterases. *Biotechnology and Bioengineering*, 113(5), 1024–1034. Retrieved from https://doi.org/https://doi.org/10.1002/bit.25874

Perz, V., Bleymaier, K., Sinkel, C., Kueper, U., Bonnekessel, M., Ribitsch, D., & Guebitz, G. M. (2016a). Substrate specificities of cutinases on aliphatic–aromatic polyesters and on their model substrates. *New Biotechnology*, 33(2), 295–304. Retrieved from https://doi.org/https://doi.org/10.1016/j.nbt.2015.11.004

Perz, V., Hromic, A., Baumschlager, A., Steinkellner, G., Pavkov-Keller, T., Gruber, K., … Guebitz, G. M. (2016c). An Esterase from Anaerobic Clostridium hathewayi Can Hydrolyze Aliphatic–Aromatic Polyesters. *Environmental Science & Technology*, 50(6), 2899–2907. Retrieved from https://doi.org/10.1021/acs.est.5b04346

Shah, A. A., Kato, S., Shintani, N., Kamini, N. R., & Nakajima-Kambe, T. (2014). Microbial degradation of aliphatic and aliphatic-aromatic co-polyesters. *Applied Microbiology and Biotechnology*, 98(8), 3437–3447. Retrieved from https://doi.org/10.1007/s00253-014-5558-1

Simões, L. C., Simões, M., & Lima, N. (2015). Kinetics of biofilm formation by drinking water isolated Penicillium expansum. *Biofouling*, 31(4), 349–362. Retrieved from https://doi.org/10.1080/08927014.2015.1042873

Soulenthone, P., Tachibana, Y., Muroi, F., Suzuki, M., Ishii, N., Ohta, Y., & Kasuya, K. (2020). Characterization of a mesophilic actinobacteria that degrades poly(butylene adipate-co-terephthalate). *Polymer Degradation and Stability*, 181, 109335. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2020.109335

Soulenthone, P., Tachibana, Y., Suzuki, M., Mizuno, T., Ohta, Y., & Kasuya, K. (2021). Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the mesophilic actinobacteria Rhodococcus fascians. *Polymer Degradation and Stability*, 184, 109481. Retrieved from https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2021.109481

Suzuki, K., Noguchi, M. T., Shinozaki, Y., & Koitabashi, M. (2014). Purification , characterization , and cloning of the gene for a biodegradable plastic-degrading enzyme from Paraphoma -related fungal strain B47-9, 4457–4465. Retrieved from https://doi.org/10.1007/s00253-013-5454-0

Thumarat, U., Kawabata, T., Nakajima, M., Nakajima, H., Sugiyama, A., Yazaki, K., … Kawai, F. (2015). Comparison of genetic structures and biochemical properties of tandem cutinase-type polyesterases from Thermobifida alba AHK119. *Journal of Bioscience and Bioengineering*, 120(5), 491–497. Retrieved from https://doi.org/10.1016/j.jbiosc.2015.03.006

Thumarat, U., Nakamura, R., Kawabata, T., Suzuki, H., & Kawai, F. (2012). Biochemical and genetic analysis of a cutinase-type polyesterase from a thermophilic Thermobifida alba AHK119. *Applied Microbiology and Biotechnology*, 95(2), 419–430. Retrieved from https://doi.org/10.1007/s00253-011-3781-6

Trinh Tan, F., Cooper, D. G., Marić, M., & Nicell, J. A. (2008). Biodegradation of a synthetic co-polyester by aerobic mesophilic microorganisms. *Polymer Degradation and Stability*, 93(8), 1479–1485. Retrieved from https://doi.org/10.1016/j.polymdegradstab.2008.05.005

Van Krevelen, D. W., & Te Nijenhuis, K. (2009). Polymer Properties. In D. W. Van Krevelen & K. B. T.-P. of P. (Fourth E. Te Nijenhuis (Eds.) (pp. 3–5). Amsterdam: Elsevier. Retrieved from https://doi.org/https://doi.org/10.1016/B978-0-08-054819-7.00001-7

Wallace, P. W., Haernvall, K., Ribitsch, D., Zitzenbacher, S., Schittmayer, M., Steinkellner, G., … Birner-Gruenberger, R. (2017). PpEst is a novel PBAT degrading polyesterase identified by proteomic screening of Pseudomonas pseudoalcaligenes. *Applied Microbiology and Biotechnology*, 101(6), 2291–2303. Retrieved from https://doi.org/10.1007/s00253-016-7992-8

Watanabe, T., Suzuki, K., Shinozaki, Y., & Yarimizu, T. (2015). A UV-induced mutant of Cryptococcus flavus GB-1 with increased production of a biodegradable plastic-degrading enzyme. *Process Biochemistry*, 50(11), 1718–1724. Retrieved from https://doi.org/10.1016/j.procbio.2015.07.005

Witt, U., Einig, T., Yamamoto, M., Kleeberg, I., Deckwer, W. D., & Müller, R. J. (2001). Biodegradation of aliphatic-aromatic copolyesters: Evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. *Chemosphere*, 44(2), 289–299. Retrieved from https://doi.org/10.1016/S0045-6535(00)00162-4

Yamamoto, M., Witt, U., Skupin, G., Beimborn, D., & Müller, R.-J. (2005). Biodegradable Aliphatic-Aromatic Polyesters: "Ecoflex®". In *Biopolymers Online*. Retrieved from https://doi.org/https://doi.org/10.1002/3527600035.bpol4011

Zumstein, M. T., Rechsteiner, D., Roduner, N., Perz, V., Ribitsch, D., Guebitz, G. M., … Sander, M. (2017). Enzymatic Hydrolysis of Polyester Thin Films at the Nanoscale: Effects of Polyester Structure and Enzyme Active-Site Accessibility. *Environmental Science & Technology*, 51(13), 7476–7485. Retrieved from https://doi.org/10.1021/acs.est.7b01330

CHAPTER 7.
FINAL REMARKS

7.1 GENERAL CONCLUSIONS

The work presented in this thesis is the product of a systematic study aiming to analyse biodegradation in soil of PHAs/PBAT bilayer films. To achieve this goal, several aspects affecting plastic biodegradation in soil were explored, such as the films' physicochemical properties, the soil microbiome dynamics, and the influence of relevant abiotic parameters on the process. Novel microorganisms with the ability to biodegrade the polymers, including the resistant PBAT, could be successfully isolated. The main conclusions of this thesis will be summarized below.

The assessment of the film's physicochemical properties by all the characterization techniques allowed to understand some of the complex biodegradation processes. Both bilayer films tested in Chapters 3 and 4, presented similar biodegradation in soil (> 45 %), with the PHAs layers being the main contributor, due to the disappearance of the final residue. For the first time, the bilayer films of PHA/PBAT were tested in soil, and because there is 70 % of PHBV (in the PHBV/PBAT film) and 45 % of PHB (in the PHB/PBAT film) it is possible to conclude that the PHB biodegradation was faster than the PHBV, being PHB/PBAT films better alternatives and showing more biodegradation potential than PHBV/PBAT films. PBAT was biodegraded to a lesser extent in both films (as expected). The PHBV/PBAT film was surrounded by a network of fungal hyphae filaments, detected by SEM analysis, suggesting that fungi played a vital role in the biodegradation process.

The clear-zone technique was once again proven to be useful in the isolation of polymer degradation microorganisms since several fungi and bacteria were isolated with the ability to biodegrade PHBV and PHB. Remarkably, microorganisms with the ability to biodegrade PBAT were isolated as well. These were fungi closely related to *Aspergillus pseudodeflectus* and *Purpureocillium lilacinum*. Only a strain of *Purpureocillium lilacinum*, different from the one isolated in this work, has already been identified as PBAT degrader, and no microorganisms assigned to *Aspergillus pseudodeflectus* have been indicated with this capability thus far. Due to the PHBV/PBAT decomposition, significant differences were detected in the taxonomic diversity of prokaryotic and eukaryotic populations before and after the biodegradation experiment. Furthermore, microorganisms assigned to *Aspergillus pseudodeflectus* and *Purpureocillium lilacinum* could be detected in the soil used in the PHBV/PBAT biodegradation experiments. This, suggests that it is possible that these microorganisms have been also contributing to the PBAT degradation in those experiments, although they could not be isolated from the soil in that specific experiment.

The CCRD approach revealed that the temperature was the only significant independent variable, with higher temperatures (37 - 45 °C) improving the response variable, biodegradation (of PHB/PBAT films). The 60 % moisture holding capacity produced analogous outcomes to the range normally recommended by the standard methodology (80 % and 100 %), suggesting the widening of the range. The addition or not of nitrogen to the soil, in relation to the carbon existing in the film, did not present significant differences. Although the addition of nitrogen is required by the ASTM D5988, to guarantee that nitrogen is present and is not a limiting factor, since N is necessary for the biodegradation of C, it seems in fact that the initial C:N ratio existing in the soil was a more relevant factor. Based on the results, the influence of the soil C:N ratio should be studied for the development of new soil standards, possibly considering the development of C:N ratio ranges for soils. The prediction model for the biodegradation of PHB/PBAT films in similar soils obtained a very high correlation between the estimated and experimental results of 0.99. This result indicates that the model developed can thus be used as a reliable tool for the simulation of this film biodegradation in comparable soils and within the range of the parameters tested.

The BOD demonstrated that isolate 7 (assigned to *Purpureocillium lilacinum*) was more efficient to biodegrade PBAT than isolate 9 (assigned to *Aspergillus pseudodeflectus*) because it degraded 11 % more PBAT. Thus far, to our knowledge, no microorganisms were reported as capable of degrading both PBAT and PHB (apart from the two microorganisms isolated here) under the tested conditions.

Overall and even considering that several parallel or complementary works could and should be performed, it is safe to conclude that PHAs/PBAT bilayer films are a much better choice from an environmental point of view than the ubiquitous conventional plastics used indiscriminately throughout the history of plastics' utilization. The results of this work indicate that the PHAs degraded at a fast rate, while the presence of PBAT considerably slowed down the biodegradation process at mesophilic temperatures, however, it seems that it would eventually degrade suggesting that they would not accumulate in a soil environment. The PBAT displayed great potential to be biodegraded in a relatively short period of time in environmentally relevant conditions, especially when compared with most of the polymers extensively used. This work is highly relevant as it increased knowledge about PHAs/PBAT films biodegradation in soil and contributed to new strains with the capacity to degrade PBAT. This knowledge and these microorganisms and/or their enzymes could be applied in efficient biotechnological solutions for the treatment of waste containing PBAT, at mesophilic temperatures, thus contributing to environmental cleaning and a

circular economy. Furthermore, if the films are contaminated by residues, and is not possible to recover/recycle them, and considering their properties, these films are good candidates for other end-of-life routes such as industrial or home composting. It is of utmost importance to test these options, which are much better than incineration or using landfills which are common strategies to deal with conventional plastics in today's society.

7.2 GUIDELINES FOR FUTURE WORK

The results from this work provided very important insights into the biodegradation of PHAs/PBAT films in soils. Despite the findings of this thesis, some work still needs to be done to fully understand the potential and minimize risks of using these films as a replacement for conventional plastics. Although these materials are usually considered harmless individually, ecotoxicological testing of these films should be performed, to elucidate the environmental impact not only on plants and invertebrates but also on microorganisms that are often overlooked.

It would be interesting to test the final packaging applications since properties such as thickness and surface area (among others) are known to be factors that influence the biodegradation process. The CCDR approach allowed to analyse the effect of different factors on the biodegradation of the PHB/PBAT films in soil. Nonetheless, other factors, including nutrients and type of soil, could also be included to test their impact and ultimately improve the model. After (un)intentional release, it is common for plastic to suffer weathering (such as exposure to UV light), with considerable impacts on its properties. In this sense, these factors could also be included in the CCDR approach.

Given the potential of these microorganisms (isolates 7 and 9) more research should be done towards finding the ideal growth conditions (nitrogen source, pH ...) to degrade more efficiently, PBAT. In addition, more work should be done to confirm which enzymes are being expressed during biodegradation (which can be assessed by doing transcriptomics tests during biodegradation). To check which ester bonds the enzymes of these microorganisms are capable of cleaving, chromatography techniques such as HPLC and GC-MS should be applied. In order to meet the European recommendations for a circular economy, and within the scope of recycling or management of environments with mixed plastic wastes, the enzymes isolation and the testing of enzymatic technology could eventually be more efficient than the use of microorganisms. The microorganisms or enzymes could be used for the monomer recycling process, reducing the need for new production of biopolymer building blocks. The bioaugmentation of these microorganisms in soils contaminated with PBAT could also be an interesting strategy to accelerate *in situ* biodegradation, for plastics-contaminated ecosystems.

Due to the possibility of plastics ending up in different types of environments, it would be of utmost importance to carry out a multitude of tests before considering a material as biodegradable. Given the difficulty of biodegrading plastics in aquatic environments due to the lower amounts of microbes present, it would be imperative to test these films in this type of environment. Performing field

biodegradation testing in different sites, with several types of soils and climates is also crucial to better understand the biodegradation of this material in soil

APPENDICES

Appendix from Chapter 3

Table S1 Taxonomic identification of the *Bacteria* and *Archaea* phyla identified by the 16S rRNA gene with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (phyla)	Relative abundance (%)	
	Inoculum	After Incubation
Acidobacteria	7.55 ± 2.60	9.97 ± 3.44
Actinobacteria	41.46 ± 6.64	19.84 ± 3.18
Bacteroidetes	3.57 ± 0.57	2.04 ± 0.33
Chloroflexi	1.73 ± 0.14	2.87 ± 0.23
Firmicutes	3.80 ± 0.57	7.60 ± 1.14
Planctomycetes	3.20 ± 0.45	4.43 ± 0.63
Proteobacteria	28.04 ± 0.42	31.17 ± 0.47
Unclassified Bacteria	4.95 ± 0.21	7.50 ± 0.31
Verrucomicrobia	1.65 ± 0.20	2.42 ± 0.29
Gemmatimonadetes	0.69 ± 0.01	2.07 ± 0.03
Crenarchaeota	1.20 ± 0.15	4.54 ± 0.58
Unclassified Archaea	0.13 ± 0.02	1.18 ± 0.14
Others	2.15 ± 0.43	2.70 ± 0.54
No Hit	0.69 ± 0.10	1.68 ± 0.25

Table S2 Taxonomical classification of the phylum (*Bacteria* or *Achaea*) that have statistically significant differences in the soil before and after adding the PHBV/PBAT film ($p < 0.05$)

Taxonomic identification	Relative abundance (%)		Ratio
	Inoculum	After Incubation	
Fusobacteria	< 0.01 ± < 0.01	0.08 ± 0.02	25.53
Unclassified Archaea	0.13 ± 0.02	1.18 ± 0.14	9.11
Chlorobi	0.05 ± 0.00	0.21 ± 0.02	4.09
Crenarchaeota (Archaea)	1.20 ± 0.15	4.54 ± 0.58	3.80
Gemmatimonadetes	0.69 ± 0.01	2.07 ± 0.03	2.98
Chloroflexi	1.73 ± 0.14	2.87 ± 0.23	1.66
Unclassified Bacteria	4.95 ± 0.21	7.50 ± 0.31	1.51
Proteobacteria	28.04 ± 0.42	31.17 ± 0.47	1.11
Tenericutes	0.08 ± 0.02	0.01 ± 0.00	0.11

Table S3 Taxonomic identification of the phyla identified by the 18S rRNA gene with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (phyla)	Relative abundance (%)	
	Inoculum	After Incubation
Proteobacteria	11.99 ± 4.99	5.70 ± 2.37
Apicomplexa	1.71 ± 0.26	1.36 ± 0.21
Unclassified *Eukaryota*	6.08 ± 1.57	8.24 ± 2.12
Ascomycota	45.67 ± 6.06	16.72 ± 2.22
Basidiomycota	12.75 ± 3.80	5.92 ± 1.76
Unclassified *Fungi*	2.24 ± 0.49	2.43 ± 0.54
No Hit	10.94 ± 2.76	49.81 ± 1.38
Chlorophyta	2.48 ± 1.16	1.41 ± 0.66
Streptophyta	0.52 ± 0.00	3.96 ± 0.69
Unknown	0.92 ± 0.00	1.11 ± 0.06
Others	18.11 ± 2.11	11.82 ± 1.38

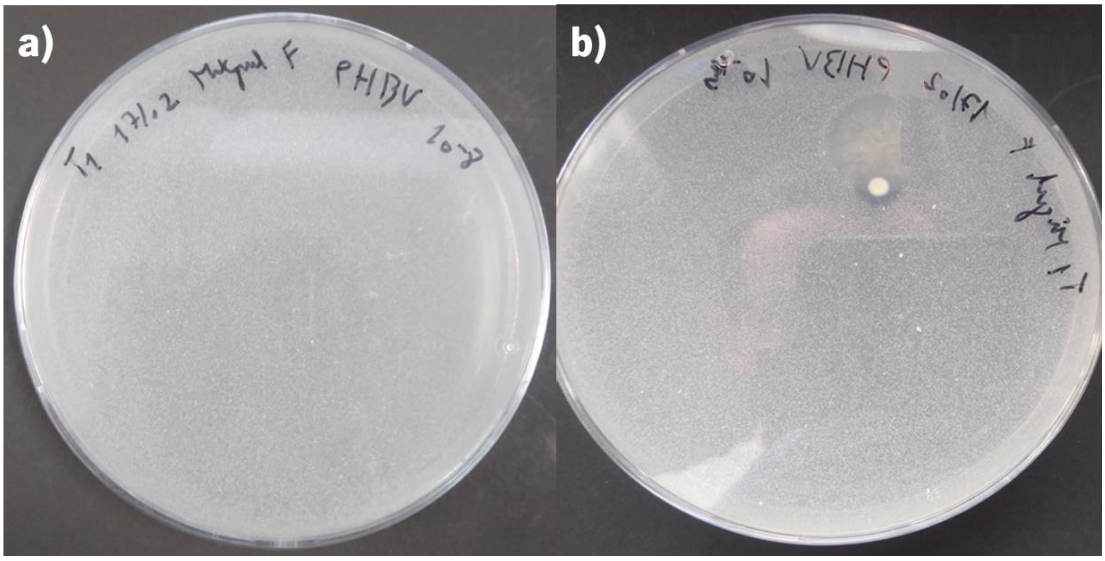

Figure S1 PHBV plates, a) without microorganisms, b) With a bacterial colony.

Appendix from Chapter 4

Table S1 Taxonomical identification of the soil prokaryotic microorganisms with the biggest differences in terms of relative abundance ($p < 0.05$)

Taxonomic identification (Species)	Relative abundance (%)		Ratio
	Inoculum	After Incubation	
Variovorax defluvii	0.11 ± 0.01	0.03 ± < 0.01	0.29
Pseudomonas sp	1.88 ± 0.14	0.16 ± 0.03	0.09
Rhodoferax sp	0.02 ± < 0.01	< 0.01 ± < 0.01	0.04
Phytoplasma sp	0.02 ± < 0.01	< 0.01 ± < 0.01	0.04
Taxonomic identification (Genera)	**Inoculum**	**After Incubation**	**Ratio**
Pseudomonas	1.88 ± 0.14	0.16 ± 0.03	0.09
Acinetobacter	0.03 ± < 0.01	< 0.01 ± < 0.01	0.06
Phormidium	0.01 ± < 0.01	< 0.01 ± < 0.01	0.06
Rhizobacter	0.15 ± 0.02	0.01 ± 0.01	0.05
Rhodoferax	0.02 ± < 0.01	< 0.01 ± < 0.01	0.04
Segetibacter	0.08 ± < 0.01	< 0.01 ± < 0.01	0.04
Clostridiisalibacter	< 0.01 ± 0.01	< 0.01 ± < 0.01	0.04
Candidatus Phytoplasma	0.02 ± < 0.01	< 0.01 ± < 0.01	0.04
Aquabacterium	0.06 ± 0.01	< 0.01 ± < 0.01	0.04
Marinobacter	0.02 ± 0.01	< 0.01 ± < 0.01	0.03
Acidovorax	0.01 ± < 0.01	< 0.01 ± < 0.01	0.03
Pantoea	0.01 ± 0.01	< 0.01 ± < 0.01	0.03
Georgfuchsia	0.01 ± 0.00	< 0.01 ± < 0.01	0.03
Acidocella	0.01 ± 0.01	< 0.01 ± < 0.01	0.02
Roseimicrobium	0.03 ± 0.02	< 0.01 ± < 0.01	0.01
Duganella	0.38 ± 0.04	< 0.01 ± < 0.01	0.01
Luteimonas	0.05 ± 0.06	< 0.01 ± < 0.01	0.01

*Ratio – relative abundance after incubation / relative abundance in the inoculum

Table S2 Taxonomic identification of the Bacteria and Archaea phyla identified by the 16S rRNA gene with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (phyla)	Relative abundance (%)	
	Inoculum	After Incubation
Crenarchaeota	2.42 ± 0.20	4.50 ± 2.08
Unclassified *Archaea*	0.18 ± 0.02	1.17 ± 0.86
Proteobacteria	32.61 ± 0.50	27.31 ± 3.00
Actinobacteria	21.22 ± 1.43	23.30 ± 7.93
Acidobacteria	10.74 ± 0.32	7.43 ± 2.18
Unclassified *Bacteria*	7.55 ± 0.15	9.76 ± 1.08
Firmicutes	7.12 ± 0.79	7.81 ± 3.47
Planctomycetes	4.35 ± 0.34	4.58 ± 1.26
Bacteroidetes	3.94 ± 0.43	2.20 ± 0.93
Verrucomicrobia	2.97 ± 0.30	2.70 ± 0.91
Chloroflexi	2.80 ± 0.15	3.56 ± 0.34
Gemmatimonadetes	0.90 ± 0.02	1.41 ± 0.31
No Hit	1.40 ± 0.15	1.98 ± 0.26
Others	2.88 ± 0.41	2.26 ± 0.69

Table S3 Taxonomic identification of the phyla identified by the 18S rRNA gene with the mean relative abundance superior to 1 % in the soil before and after the biodegradation of PHB/PBAT film

Taxonomic identification (phyla)	Relative abundance (%)	
	Inoculum	After Incubation
Nematoda	1.28 ± 0.67	0.11 ± 0.11
Arthropoda	0.97 ± 0.72	6.12 ± 5.38
Unclassified *Eukaryota*	17.83 ± 0.88	17.37 ± 8.88
Apicomplexa	6.87 ± 0.49	3.45 ± 0.57
Unclassified Fungi	5.21 ± 0.34	4.81 ± 2.34
Basidiomycota	5.68 ± 0.32	7.13 ± 2.96
Ascomycota	24.59 ± 1.11	17.87 ± 3.86
Chlorophyta	1.26 ± 0.26	0.88 ± 0.11
No Hit	25.28 ± 1.60	35.53 ± 5.31
Others	11.02 ± 1.17	7.71 ± 0.75

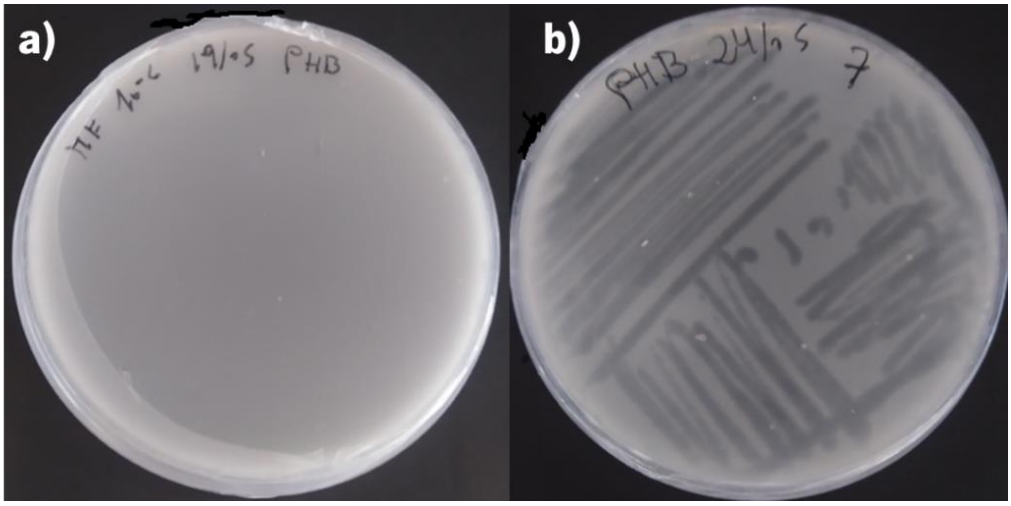

Figure S1 PHB plates, a) without microorganisms, b) With PHB isolate 7.

Appendix from Chapter 5

Table S1 Biodegradation of the control (cellulose) in the sixth month for each DoE trial. (*) - The 100 % was achieved in 89 days. The validation was performed in triplicate

Trials	C:N ratio	T (°C)	Soil Moisture Content (%)	Biodegradation (%)*
1	8.1	16.3	68.1	71.34
2	31.9	16.3	68.1	75.11
3	8.1	37.7	68.1	62.60
4	31.9	37.7	68.1	71.57
5	8.1	16.3	91.9	84.67
6	31.9	16.3	91.9	76.86
7	8.1	37.7	91.9	64.11
8	31.9	37.7	91.9	64.88
9	0	27	80	76.84
10	40	27	80	81.79
11	20	9	80	54.72
12	20	45	80	100*
13	20	27	60	69.55
14	20	27	100	80.23
15	20	27	80	72.10
16	20	27	80	89.80
17	20	27	80	74.54
18	20	27	80	73.62
19	20	27	80	76.00
Validation	20	37.7	80	80.30

*Biodegradation after 6 months

Appendix from Chapter 6

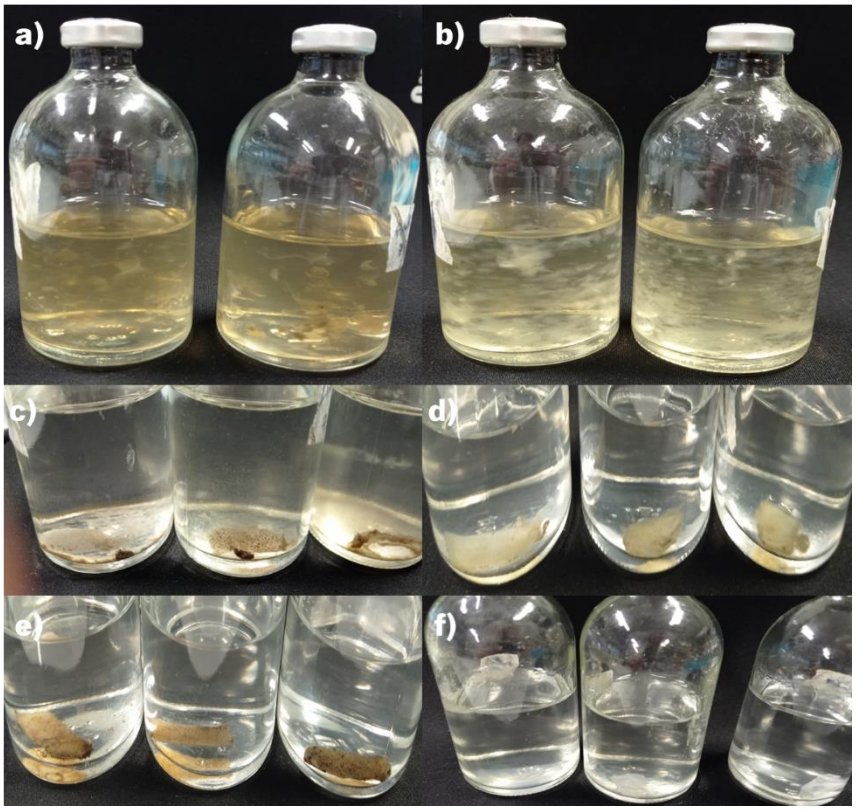

Figure S1 Glass bottles at the end of the BOD experiment. a) positive control *Aspergillus*, b) positive control *Purpureocillium lilacinum*, c) PBAT film incubated with *Aspergillus* spores, d) PBAT film incubated with *Purpureocillium lilacinum* spores, e) PBAT film incubated with *Aspergillus and Purpureocillium lilacinum* spores, f) Negative control.

Table S1 Enzymes from *Aspergillus* (tax ID 5052) identified with the models as potential PBAT degrading enzymes

Aspergillus				
Uniprot ID	**Protein names**	**Gene Names**	**Microorganism**	**Number of HMMs that identify the protein**
A0A0S7DIL9	Acyltransferase LovD	ALT_007513 ALT_1039 CNMCM8694_000671 CNMCM8927_000961 IFM58399_09935	*Aspergillus lentulus*	2
A0A0S7DM50	Acyltransferase LovD	ALT_004496 ALT_2338	*Aspergillus lentulus*	2
A0A401KPS9	Acyltransferase LovD	AAWM_04188	*Aspergillus awamori* (Black koji mold)	2
A0A0S7E3V8	Acyltransferase LovD	ALT_005586 ALT_6577	*Aspergillus lentulus*	2

A0A8H3NPK1	Acyltransferase LovD	IFM58399_03661	*Aspergillus lentulus*	2
A0A8H3NSS9	Acyltransferase LovD	IFM58399_05347	*Aspergillus lentulus*	2
A0A0S7EAM4	Acyltransferase LovD	ALT_009104 ALT_9162	*Aspergillus lentulus*	2
A0A401KQW7	Acyltransferase LovD	AAWM_04571	*Aspergillus awamori* (Black koji mold)	1
Q4X078	Cutinase	AFUA_2G14420	*Aspergillus fumigatus* (strain ATCC MYA-4609 / CBS 101355 / FGSC A1100 / Af293) *(Neosartorya fumigata)*	10
B0XU21	Cutinase	AFUB_030040	*Aspergillus fumigatus* (strain CBS 144.89 / FGSC A1163 / CEA10) *(Neosartorya fumigata)*	10
A0A0J5Q122	Cutinase	Y699_02431	*Aspergillus fumigatus* Z5	10
A0A229XEA7	Cutinase	CDV57_08447	*Aspergillus fumigatus (Neosartorya fumigata)*	10
A0A8H4HRU2	Cutinase	CNMCM8057_000710 CNMCM8686_001394	*Aspergillus fumigatus (Neosartorya fumigata)*	10
A0A2I1CNR1	Cutinase	P174DRAFT_416947	*Aspergillus novofumigatus* (strain IBT 16806)	11
A0A8E0QLK6	Cutinase	Aud_001396	*Aspergillus udagawae*	11
A0A8H3P8C7	Cutinase	IFM46972_07634	*Aspergillus udagawae*	11
A0A8H3RNC9	Cutinase	IFM51744_02137	*Aspergillus udagawae*	11
A1DHZ6	Cutinase	NFIA_089600	*Neosartorya fischeri* (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) *(Aspergillus fischerianus)*	10
A0A0S7DLJ4	Cutinase	ALT_000413 ALT_3654 CNMCM8927_005995 IFM58399_01988	*Aspergillus lentulus*	11
A0A8H4DS61	Cutinase	CNMCM8694_006007	*Aspergillus lentulus*	10
A0A8H4GLD0	Cutinase	CNMCM6457_009834 CNMCM6805_008258	*Aspergillus fumigatiaffinis*	10
A0A8H6VF49	Cutinase	CNMCM7691_005853	*Aspergillus felis*	10
A0A8H6QJA3	Cutinase	CNMCM5623_006340	*Aspergillus felis*	10
A0A397HAF0	Cutinase	CDV56_103901	*Aspergillus thermomutatus*	10
A0A397IAC0	Cutinase	CDV55_105853	*Aspergillus turcosus*	10

A0A8H6PSV7	Cutinase	CNMCM5793_008719 CNMCM6106_007116	*Aspergillus hiratsukae*	10
A0A5N5X5V7	Cutinase	BDV29DRAFT_115338	*Aspergillus leporis*	9
A0A5N6VZP6	Cutinase	BDV41DRAFT_577530	*Aspergillus transmontanensis*	9
A0A5N6DS80	Cutinase	BDV34DRAFT_222845	*Aspergillus parasiticus*	9
A0A1F8AGU1	Cutinase	ABOM_000098	*Aspergillus bombycis*	9
A0A5N6I1F2	Cutinase	BDV32DRAFT_150394	*Aspergillus pseudonomiae*	9
A0A5N7DF90	Cutinase	BDV37DRAFT_282875	*Aspergillus pseudonomiae*	9
A0A5N6V3B7	Cutinase	BDV40DRAFT_297494	*Aspergillus tamarii*	9
A0A5N6HAQ8	Cutinase	BDV35DRAFT_388317	*Aspergillus flavus*	9
A0A1S9DXD5	Cutinase	OAory_01023230	*Aspergillus oryzae* (Yellow koji mold)	8
A0A2G7FNS7	Cutinase	AARAC_000081	*Aspergillus arachidicola*	8
A0A5N6SX09	Cutinase	BDV38DRAFT_242995	*Aspergillus pseudotamarii*	9
A0A2V5J821	Cutinase	BP00DRAFT_390567	*Aspergillus indologenus* CBS 114.80	10
A0A1L9R9P1	Cutinase	ASPWEDRAFT_698165	*Aspergillus wentii* DTO 134E9	10
A0A5N6G778	Cutinase	BDW43DRAFT_306927	*Petromyces alliaceus (Aspergillus alliaceus)*	9
A0A8H5ZVY6	Cutinase	ETB97_007710	*Aspergillus burnettii*	9
A0A7U2QUE1	Cutinase	F9C07_2147579	*Aspergillus flavus*	9
A0A5N7EMN1	Cutinase	BDV36DRAFT_294153	*Aspergillus pseudocaelatus*	9
A0A3M7JPB3	Cutinase	CA14_003198	*Aspergillus flavus*	9
A0A5N6JC83	Cutinase	BDV30DRAFT_236157	*Aspergillus minisclerotigenes*	9
A0A017SIZ4	Cutinase	EURHEDRAFT_475414	*Aspergillus ruber* (strain CBS 135680)	11
Q0CZ47	cutinase	ATEG_01037	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	9
A0A317UNN0	Cutinase	BO83DRAFT_442457	*Aspergillus eucalypticola* (strain CBS 122712 / IBT 29274)	8
A0A5M3YKQ3	cutinase	ATEIFO6365_0001004900 ATETN484_0001004800	*Aspergillus terreus*	9
A0A8G1RUC4	Cutinase	BO72DRAFT_428459	*Aspergillus fijiensis* CBS 313.89	10
A0A0L1INP6	Cutinase	ANOM_010417	*Aspergillus nomiae* NRRL 13137	9

B8N7P9	Cutinase	AFLA_104920 G4B84_004266	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	8
A0A2P2HB95	Cutinase	AFLA70_50g003671	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	8
A0A318ZBM0	Cutinase	BP01DRAFT_343650	*Aspergillus saccharolyticus* JOP 1030-1	8
A0A1L9WKG0	Cutinase	ASPACDRAFT_63532	*Aspergillus aculeatus* (strain ATCC 16872 / CBS 172.66 / WB 5094)	9
A0A1E3BKU6	Cutinase	SI65_04059	*Aspergillus cristatus* (Chinese Fuzhuan brick tea-fermentation fungus) *(Eurotium cristatum)*	11
A0A7R7VRR7	Cutinase	ACHE_50821S	*Aspergillus chevalieri (Eurotium chevalieri)*	10
A0A5N6TL99	Cutinase	BDV25DRAFT_169396	*Aspergillus avenaceus*	9
A0A319C9I2	Cutinase	BO82DRAFT_382954	*Aspergillus uvarum* CBS 121591	9
A0A319F5Q5	Cutinase	BO78DRAFT_434338	*Aspergillus sclerotiicarbonarius* (strain CBS 121057 / IBT 28362)	8
A0A395GN85	Cutinase	BO80DRAFT_416056	*Aspergillus ibericus* CBS 121593	8
A0A8T8X6B2	Cutinase	BO86DRAFT_335953	*Aspergillus japonicus* CBS 114.51	10
A0A395I6L6	Cutinase	BO97DRAFT_387915	*Aspergillus homomorphus* (strain CBS 101889)	8
A0A318Z6C0	Cutinase	BP01DRAFT_351558	*Aspergillus saccharolyticus* JOP 1030-1	8
A0A5N7BW16	Cutinase	BDV23DRAFT_175776	*Petromyces alliaceus (Aspergillus alliaceus)*	9
A0A317WZJ9	Cutinase	BO94DRAFT_534415	*Aspergillus sclerotioniger* CBS 115572	8
Q2UIH1	Cutinase	AO090023000058	*Aspergillus oryzae* (strain ATCC 42149 / RIB 40) (Yellow koji mold)	9
A0A5N7ALU7	Cutinase	BDV27DRAFT_140868	*Aspergillus caelatus*	9

A0A1L9UYJ1	Cutinase	ASPBRDRAFT_145740	*Aspergillus brasiliensis* (strain CBS 101740 / IMI 381727 / IBT 21946)	8
A0A505ILZ7	Cutinase	CAN33_009470	*Aspergillus niger*	8
A0A8G1R4U5	Cutinase	BO85DRAFT_416677	*Aspergillus piperis* CBS 112811	8
A0A5N6YNI7	Cutinase	BDV24DRAFT_148516	*Aspergillus arachidicola*	9
A0A5N6XMQ8	Cutinase	BDV39DRAFT_188320	*Aspergillus sergii*	9
A0A319BH53	Cutinase	BO88DRAFT_363061	*Aspergillus vadensis* (strain CBS 113365 / IMI 142717 / IBT 24658)	8
A0A318ZG12	Cutinase	BP01DRAFT_346805	*Aspergillus saccharolyticus* JOP 1030-1	8
A0A1M3TXP5	Cutinase	ASPFODRAFT_66149	*Aspergillus luchuensis* (strain CBS 106.47)	8
A0A100I5Q6	Cutinase	ABL_01103	*Aspergillus niger*	8
A0A1L9N9N6	Cutinase	ASPTUDRAFT_442563	*Aspergillus tubingensis* (strain CBS 134.48)	8
A0A317UKG8	Cutinase	BO83DRAFT_421932	*Aspergillus eucalypticola* (strain CBS 122712 / IBT 29274)	8
A0A5N6F115	Cutinase	BDV33DRAFT_228983	*Aspergillus novoparasiticus*	9
A0A318YF06	Cutinase	BO87DRAFT_362747	*Aspergillus neoniger* (strain CBS 115656)	8
A0A8H3XZG7	Cutinase	AtWU_06277	*Aspergillus tubingensis*	8
A0A146G171	Cutinase	AKAW2_21545A ALUC_21481A RIB2604_04200270	*Aspergillus kawachii* (White koji mold) *(Aspergillus awamori var. kawachi)*	8
G7Y0V7	Cutinase	AKAW_10929	*Aspergillus kawachii (strain NBRC 4308) (White koji mold) (Aspergillus awamori var. kawachi)*	8
A0A2I2G3D5	Cutinase	P170DRAFT_413282	*Aspergillus steynii* IBT 23096	8
A0A2V5HK64	Cutinase	BO99DRAFT_452429	*Aspergillus violaceofuscus* (strain CBS 115571)	9
A0A370PJP5	Cutinase	M752DRAFT_301489	*Aspergillus phoenicis* ATCC 13157	8
A0A401KHR4	Cutinase	AAWM_01789	*Aspergillus awamori* (Black koji mold)	8

A0A319B5W0	Cutinase	BO96DRAFT_472046	*Aspergillus lacticoffeatus* (strain CBS 101883)	8
A0A370CEI8	Cutinase	M747DRAFT_336820	*Aspergillus niger* ATCC 13496	8
A2QBP1	Cutinase	An02g00730	*Aspergillus niger* (strain ATCC MYA-4892 / CBS 513.88 / FGSC A1513)	8
A0A1R3RXX3	Cutinase	ASPCADRAFT_160089	*Aspergillus carbonarius* (strain ITEM 5010)	8
A0A0R6I0L5	Cutinase	ATCC64974_59570	*Aspergillus niger*	8
A0A3F3PYY9	Cutinase	BDQ94DRAFT_171395	*Aspergillus welwitschiae*	8
A0A4S3JLE8	Cutinase	ATNIH1004_008129 EYZ11_006410	*Aspergillus tanneri*	7
A0A0U5GG86	Cutinase	ASPCAL13480	*Aspergillus calidoustus*	7
G3Y192	Cutinase	ASPNIDRAFT_36638	*Aspergillus niger* (strain ATCC 1015 / CBS 113.46 / FGSC A1144 / LSHB Ac4 / NCTC 3858a / NRRL 328 / USDA 3528.7)	9
A0A0F8V3J9	cutinase	ARAM_001573	*Aspergillus rambellii*	7
A0A0F8VN30	cutinase	AOCH_001615	*Aspergillus ochraceoroseus*	7
A0A5N6ZFL7	cutinase	BDV28DRAFT_127276	*Aspergillus coremiiformis*	9
A0A1L9VMV4	Cutinase	ASPGLDRAFT_148132	*Aspergillus glaucus* CBS 516.65	6
A0A317VQY2	Cutinase	BO70DRAFT_364294	*Aspergillus heteromorphus* CBS 117.55	8
A0A2T5LVW1	cutinase	P175DRAFT_0481787	*Aspergillus ochraceoroseus* IBT 24754	7
A0A319D0D1	Cutinase	BO71DRAFT_387060	*Aspergillus ellipticus* CBS 707.79	7
A0A1L9PYQ2	Cutinase	ASPVEDRAFT_140732	*Aspergillus versicolor* CBS 583.65	8
A0A7R8AV91	Cutinase	APUU_80956A	*Aspergillus puulaauensis*	8
Q5B9E7	Cutinase	AN2833.2	*Emericella nidulans* (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) *(Aspergillus nidulans)*	7
A0A3D8Q9R2	Cutinase	DSM5745_11257	*Aspergillus mulundensis*	6
A0A317UWH6	Cutinase	BO83DRAFT_320961	*Aspergillus eucalypticola* (strain CBS 122712 / IBT 29274)	6

A0A0U5GH79	cutinase	ASPCAL14489	*Aspergillus calidoustus*	2
A0A5N6U914	Cutinase	BDV25DRAFT_146566	*Aspergillus avenaceus*	19
A0A5N5WTX2	Cutinase	BDV29DRAFT_197569	*Aspergillus leporis*	23
A0A5N6SDI5	Cutinase	BDV38DRAFT_199823	*Aspergillus pseudotamarii*	14
A0A5N7A336	Cutinase	BDV27DRAFT_118143	*Aspergillus caelatus*	14
A0A5N6UF70	Cutinase	BDV40DRAFT_59575	*Aspergillus tamarii*	14
A0A5N6Z6B7	Cutinase	BDV28DRAFT_157229	*Aspergillus coremiiformis*	10
A0A8H6A5Y5	Cutinase	ETB97_000514	*Aspergillus burnettii*	15
A0A5N7C5F6	Cutinase	BDV23DRAFT_184574	*Petromyces alliaceus (Aspergillus alliaceus)*	13
A0A1F8A0U7	Cutinase	ABOM_005574	*Aspergillus bombycis*	14
A0A2G7FU55	Cutinase	AARAC_011301 BDV24DRAFT_139032	*Aspergillus arachidicola*	15
A0A7U2MHH0	Cutinase	F9C07_1715	*Aspergillus flavus*	15
B8N2Q5	Cutinase	AFLA_023390 G4B84_004106	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	15
A0A2P2H6J5	Cutinase	AFLA70_20g004971	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	15
A0A364MJI3	Cutinase	CA14_011528	*Aspergillus flavus*	15
I8IRG2	Cutinase	Ao3042_01510	*Aspergillus oryzae* (strain 3.042) (Yellow koji mold)	15
Q2UIR7	Cutinase	AO090003001507	*Aspergillus oryzae* (strain ATCC 42149 / RIB 40) (Yellow koji mold)	15
Q9P960	Cutinase	tglA	*Aspergillus oryzae* (Yellow koji mold)	15
A0A5N6JMU7	Cutinase	BDV30DRAFT_232954	*Aspergillus minisclerotigenes*	16
A0A5N6GWW0	Cutinase	BDV35DRAFT_380697	*Aspergillus flavus*	14
A0A5N6X7H2	Cutinase	BDV39DRAFT_78727	*Aspergillus sergii*	16
A0A0F0IG15	Cutinase	P875_00010039	*Aspergillus parasiticus* (strain ATCC 56775 / NRRL 5862 / SRRC 143 / SU-1)	16
A0A5N6E2P0	Cutinase	BDV34DRAFT_85893	*Aspergillus parasiticus*	16
A0A5N6ERG8	Cutinase	BDV33DRAFT_100406	*Aspergillus novoparasiticus*	16
A0A5N6VPA4	Cutinase	BDV41DRAFT_397207	*Aspergillus transmontanensis*	16

A0A0M4APK0	Cutinase		*Aspergillus oryzae* (Yellow koji mold)	13
A0A1S9DKL5	Cutinase	OAory_01053960	*Aspergillus oryzae* (Yellow koji mold)	13
A0A5N7BL11	Cutinase	BDV26DRAFT_4929	*Aspergillus bertholletiae*	14
A0A5N6FME4	Cutinase	BDW43DRAFT_166531	*Petromyces alliaceus (Aspergillus alliaceus)*	14
A0A5N6IDF8	Cutinase	BDV32DRAFT_75274	*Aspergillus pseudonomiae*	11
A0A0L1IZG4	cutinase	ANOM_008687	*Aspergillus nomiae* NRRL 13137	11
A0A5N7D5D0	Cutinase	BDV37DRAFT_193984	*Aspergillus pseudonomiae*	8
A0A5N7ECP0	Cutinase	BDV36DRAFT_206121	*Aspergillus pseudocaelatus*	6
A0A5N6U4L1	Cutinase	BDV25DRAFT_21475	*Aspergillus avenaceus*	43
A0A317VTY8	Cutinase	BO83DRAFT_406862	*Aspergillus eucalypticola* (strain CBS 122712 / IBT 29274)	45
A0A1R3RAC0	Cutinase	ASPCADRAFT_177434	*Aspergillus carbonarius* (strain ITEM 5010)	44
A0A146FG51	Cutinase	AKAW2_40906A ALUC_40870A RIB2604_01901460	*Aspergillus kawachii* (White koji mold) *(Aspergillus awamori var. kawachi)*	45
A0A1M3TC72	Cutinase	ASPFODRAFT_208879	*Aspergillus luchuensis* (strain CBS 106.47)	45
G7XJ51	Cutinase	AKAW_05190	*Aspergillus kawachii (strain NBRC 4308) (White koji mold) (Aspergillus awamori var. kawachi)*	45
A0A8G1VH60	Cutinase	BO85DRAFT_523893	*Aspergillus piperis* CBS 112811	45
A0A319F771	Cutinase	BO78DRAFT_381968	*Aspergillus sclerotiicarbonarius* (strain CBS 121057 / IBT 28362)	41
A0A318ZPX4	Cutinase	BP01DRAFT_294760	*Aspergillus saccharolyticus* JOP 1030-1	40
A0A2V5IJM1	Cutinase	BO99DRAFT_470243	*Aspergillus violaceofuscus* (strain CBS 115571)	41
A0A8T8XCM2	Cutinase	BO86DRAFT_354742	*Aspergillus japonicus* CBS 114.51	41
A0A2V5IJH2	Cutinase	BP00DRAFT_484704	*Aspergillus indologenus* CBS 114.80	42

A0A319CJ91	Cutinase	BO82DRAFT_351976	*Aspergillus uvarum* CBS 121591	41
A0A8G1RIE5	Cutinase	BO72DRAFT_393109	*Aspergillus fijiensis* CBS 313.89	42
A0A1L9WNQ9	Cutinase	ASPACDRAFT_62073	*Aspergillus aculeatus* (strain ATCC 16872 / CBS 172.66 / WB 5094)	41
A0A319CBV2	Cutinase	BO88DRAFT_358699	*Aspergillus vadensis* (strain CBS 113365 / IMI 142717 / IBT 24658)	45
A0A100IKI7	Cutinase	ABL_05589	*Aspergillus niger*	45
A0A8H3XT88	Cutinase	AtWU_00779	*Aspergillus tubingensis*	45
A0A1L9NKL0	Cutinase	ASPTUDRAFT_60389	*Aspergillus tubingensis* (strain CBS 134.48)	45
A0A317X6E5	Cutinase	BO94DRAFT_543182	*Aspergillus sclerotioniger* CBS 115572	43
A0A1L9UZ67	Cutinase	ASPBRDRAFT_140404	*Aspergillus brasiliensis* (strain CBS 101740 / IMI 381727 / IBT 21946)	43
A0A318Z3P7	Cutinase	BO87DRAFT_357875	*Aspergillus neoniger* (strain CBS 115656)	44
A0A395GHT6	Cutinase	BO80DRAFT_370103	*Aspergillus ibericus* CBS 121593	44
A0A254U262	Cutinase	ATCC64974_1950 CAN33_0026590	*Aspergillus niger*	44
A0A370CGU9	Cutinase	M747DRAFT_336561	*Aspergillus niger* ATCC 13496	44
A0A3F3QHS9	Cutinase	BDQ94DRAFT_166406	*Aspergillus welwitschiae*	44
A0A401KJK9	Cutinase	AAWM_02260	*Aspergillus awamori* (Black koji mold)	44
A0A317VM41	Cutinase	BO70DRAFT_364009	*Aspergillus heteromorphus* CBS 117.55	42
A0A370PET3	Cutinase	M752DRAFT_303419	*Aspergillus phoenicis* ATCC 13157	44
A0A395I9B4	Cutinase	BO97DRAFT_456352	*Aspergillus homomorphus* (strain CBS 101889)	41
A0A5N6TBW3	Cutinase	BDV38DRAFT_289560	*Aspergillus pseudotamarii*	41
A0A5N7DJC6	Cutinase	BDV32DRAFT_138656 BDV37DRAFT_292100	*Aspergillus pseudonomiae*	43
A0A2I2FW73	Cutinase	P170DRAFT_365925	*Aspergillus steynii* IBT 23096	43
A0A319CVW3	Cutinase	BO71DRAFT_364808	*Aspergillus ellipticus* CBS 707.79	42

A0A5N6G407	Cutinase	BDW43DRAFT_307378	*Petromyces alliaceus (Aspergillus alliaceus)*	43
A0A5N6ZXV4	Cutinase	BDV27DRAFT_166699	*Aspergillus caelatus*	43
A0A5N7EXS4	Cutinase	BDV36DRAFT_290144	*Aspergillus pseudocaelatus*	43
A0A2G7FR50	Cutinase	AARAC_004897 BDV24DRAFT_162691	*Aspergillus arachidicola*	43
A0A5N7C698	Cutinase	BDV23DRAFT_184241	*Petromyces alliaceus (Aspergillus alliaceus)*	43
A0A8H5ZZW0	Cutinase	ETB97_004862	*Aspergillus burnettii*	43
A0A5N6F7V2	Cutinase	BDV33DRAFT_187048	*Aspergillus novoparasiticus*	44
A0A5M3YYE0	Cutinase	ATEIFO6365_0002023200 ATETN484_0004023200	*Aspergillus terreus*	39
A0A0L1JDR3	Cutinase	ANOM_001770	*Aspergillus nomiae* NRRL 13137	44
A0A1F8A0Q8	Cutinase	ABOM_005753	*Aspergillus bombycis*	43
A0A2P2GXH1	Cutinase	AFLA70_220g001800	*Aspergillus flavus (*strain ATCC MYA-384 / AF70)	43
A0A7G5JGY8	Cutinase	G4B84_010371	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	43
A0A7U2MWK0	Cutinase	F9C07_2280507	*Aspergillus flavus*	43
A0A5N6IS50	Cutinase	BDV30DRAFT_229802	*Aspergillus minisclerotigenes*	44
A0A1L9RF54	cutinase	ASPWEDRAFT_115688	*Aspergillus wentii* DTO 134E9	35
A0A5N6Z7D8	Cutinase	BDV28DRAFT_165174	*Aspergillus coremiiformis*	37
A0A5N6HE63	Cutinase	BDV35DRAFT_403450	*Aspergillus flavus*	43
I7GSC4	Cutinase	Cut C OAory_01088120	*Aspergillus oryzae (*Yellow koji mold)	43
A0A364LUY0	Cutinase	CA14_008543	*Aspergillus flavus*	43
A0A5N6X298	Cutinase	BDV39DRAFT_192994	*Aspergillus sergii*	44
A0A0F0HZM0	Cutinase	P875_00042868	*Aspergillus parasiticus* (strain ATCC 56775 / NRRL 5862 / SRRC 143 / SU-1)	44
A0A5N6DXQ1	Cutinase	BDV34DRAFT_210310	*Aspergillus parasiticus*	44
A0A5N6VY16	Cutinase	BDV41DRAFT_564384	*Aspergillus transmontanensis*	44
A0A5N6US21	Cutinase	BDV40DRAFT_313229	*Aspergillus tamarii*	43
A0A0J5PYW8	Cutinase	Y699_02882	*Aspergillus fumigatus* Z5	37

A0A229WAI9	Cutinase	CDV57_07434 CNMCM8057_007454 CNMCM8686_007495	*Aspergillus fumigatus (Neosartorya fumigata)*	37
A0A8H4MGN0	Cutinase	CNMCM6457_007885 CNMCM6805_005550	*Aspergillus fumigatiaffinis*	37
A0A8H4G4T4	Cutinase	CNMCM8694_000453 CNMCM8927_003290 IFM58399_03875	*Aspergillus lentulus*	37
A0A0S7DWL7	Cutinase	ALT_000886 ALT_4097	*Aspergillus lentulus*	37
A0A0F8USW6	Cutinase	ARAM_007031	*Aspergillus rambellii*	36
A0A397HJ09	Cutinase	CDV56_108615	*Aspergillus thermomutatus*	38
A0A0F8UBJ4	Cutinase	AOCH_006260	*Aspergillus ochraceoroseus*	38
A0A2T5LX55	Cutinase	P175DRAFT_0523726	*Aspergillus ochraceoroseus* IBT 24754	38
A0A2I1CMD1	Cutinase	P174DRAFT_27804	*Aspergillus novofumigatus* (strain IBT 16806)	38
A0A1L9RN22	Cutinase	ASPWEDRAFT_77488	*Aspergillus wentii* DTO 134E9	34
A0A8H3NIG4	Cutinase	Aud_000922 IFM51744_01835	*Aspergillus udagawae*	37
A0A0U5GBN7	Cutinase	ASPCAL12155	*Aspergillus calidoustus*	40
A0A1L9PZ26	cutinase	ASPVEDRAFT_46049	*Aspergillus versicolor* CBS 583.65	34
A0A2I2GRY2	Cutinase	P170DRAFT_443381	*Aspergillus steynii* IBT 23096	34
A0A3M2SUH5	Cutinase	PHISP_07924	*Aspergillus* sp. HF37	38
A0A8H3NDT7	Cutinase	Aud_002246 IFM46972_02101 IFM51744_06299	*Aspergillus udagawae*	38
A0A8H6PVM7	Cutinase	CNMCM5623_006706 CNMCM7691_002210	*Aspergillus felis*	35
A0A0F8UVC6	Cutinase	AOCH_004266	*Aspergillus ochraceoroseus*	37
A0A0F8V5J0	Cutinase	ARAM_002972	*Aspergillus rambellii*	37
A0A2T5LVQ0	Cutinase	P175DRAFT_0438940	*Aspergillus ochraceoroseus* IBT 24754	37
A0A397H439	Cutinase	CDV56_103087	*Aspergillus thermomutatus*	38
A0A0S7DYM9	Cutinase	ALT_007856 ALT_4928 CNMCM8694_005293 CNMCM8927_008669 IFM58399_04546	*Aspergillus lentulus*	38
A0A8H4GRE9	Cutinase	CNMCM6805_003741	*Aspergillus fumigatiaffinis*	38
A0A8H4GGS7	Cutinase	CNMCM6457_003619	*Aspergillus fumigatiaffinis*	38
A0A421CX34	Cutinase	CFD26_102565	*Aspergillus turcosus*	37
A0A0J5SLH0	Cutinase	Y699_03577	*Aspergillus fumigatus* Z5	36

A0A229W7U0	Cutinase	CDV57_08120 CNMCM8057_003942 CNMCM8686_000009	*Aspergillus fumigatus (Neosartorya fumigata)*	36
A0A2I1C750	Cutinase	P174DRAFT_409639	*Aspergillus novofumigatus* (strain IBT 16806)	38
A0A397GD84	Cutinase	CDV55_100527	*Aspergillus turcosus*	37
A0A5M3YU02	Cutinase	ATEIFO6365_0004078500 ATETN484_0002081000	*Aspergillus terreus*	36
G3XP44	cutinase	ASPNIDRAFT_41640	*Aspergillus niger* (strain ATCC 1015 / CBS 113.46 / FGSC A1144 / LSHB Ac4 / NCTC 3858a / NRRL 328 / USDA 3528.7)	37
A0A319AE76	cutinase	BO96DRAFT_468293	*Aspergillus lacticoffeatus* (strain CBS 101883)	37
A0A0U5C405	Cutinase	ASPCAL03405	*Aspergillus calidoustus*	37
A0A5N5WS52	Cutinase	BDV29DRAFT_197958	*Aspergillus leporis*	42
A0A5N7BJN2	Cutinase	BDV26DRAFT_278383	*Aspergillus bertholletiae*	37
A0A0F8UNJ0	Cutinase	ARAM_004707	*Aspergillus rambellii*	32
A0A0F8WMR6	Cutinase	AOCH_004426	*Aspergillus ochraceoroseus*	32
A0A2T5M5W1	Cutinase	P175DRAFT_0448818	*Aspergillus ochraceoroseus* IBT 24754	32
A0A5M3Z1V7	Cutinase	ATEIFO6365_0011002300 ATETN484_0006002300	*Aspergillus terreus*	36
A0A1L9T868	cutinase	ASPSYDRAFT_48823	*Aspergillus sydowii* CBS 593.65	32
A0A1L9R4D6	Cutinase	ASPWEDRAFT_121786	*Aspergillus wentii* DTO 134E9	36
A0A1L9RPZ1	Cutinase	ASPWEDRAFT_181950	*Aspergillus wentii* DTO 134E9	31
A0A7R7XZE2	cutinase	APUU_80814A	*Aspergillus puulaauensis*	34
A0A0U5GQH5	Cutinase	ASPCAL07697	*Aspergillus calidoustus*	38
A0A2I2FIG9	Cutinase	BDW47DRAFT_134808	*Aspergillus candidus*	34
A0A2J5HR16	Cutinase	BDW42DRAFT_201594	*Aspergillus taichungensis*	34
A0A2I1D761	Cutinase	P168DRAFT_303454	*Aspergillus campestris* IBT 28561	34
A0A8H6PWL3	Cutinase	CNMCM5793_006395 CNMCM6106_008717	*Aspergillus hiratsukae*	35
A0A1E3B192	Cutinase	SI65_09909	*Aspergillus cristatus* (Chinese Fuzhuan brick tea-fermentation fungus) *(Eurotium cristatum)*	39

A0A7R7VUS8	Cutinase	ACHE_70024A	*Aspergillus chevalieri (Eurotium chevalieri)*	39
A0A0U5GGI8	Cutinase	ASPCAL14703	*Aspergillus calidoustus*	38
A0A8H6Q4X5	Cutinase	CNMCM5623_010005 CNMCM7691_002857	*Aspergillus felis*	33
A0A1L9V7P6	Cutinase	ASPGLDRAFT_1504135	*Aspergillus glaucus* CBS 516.65	39
A0A2I1C404	Cutinase	P174DRAFT_451713	*Aspergillus novofumigatus* (strain IBT 16806)	34
A0A017SR61	Cutinase	EURHEDRAFT_471081	*Aspergillus ruber* (strain CBS 135680)	38
A0A8H3S7L4	Cutinase	IFM51744_07900	*Aspergillus udagawae*	34
A0A8H3SG74	Cutinase	IFM46972_11403	*Aspergillus udagawae*	33
A0A8E0UYH2	Cutinase	Aud_006926	*Aspergillus udagawae*	34
A0A0J5PIG4	Cutinase	Y699_09222	*Aspergillus fumigatus* Z5	35
A0A229XTK6	Cutinase	CDV57_06785	*Aspergillus fumigatus (Neosartorya fumigata)*	35
A0A229Z420	Cutinase	CDV55_107184 CFD26_107225	*Aspergillus turcosus*	36
A0A8H3NCX8	Cutinase	IFM46972_02532	*Aspergillus udagawae*	33
A0A8H4HSR3	Cutinase	CNMCM8057_008080	*Aspergillus fumigatus (Neosartorya fumigata)*	34
A0A8H4MV42	Cutinase	CNMCM8686_008620	*Aspergillus fumigatus (Neosartorya fumigata)*	34
A0A8H4DB98	Cutinase	CNMCM8694_002004 CNMCM8927_004115	*Aspergillus lentulus*	34
A0A0S7DU46	Cutinase	ALT_008856 ALT_5832 IFM58399_05715	*Aspergillus lentulus*	34
A0A1L9RZ92	Cutinase	ASPWEDRAFT_144666	*Aspergillus wentii* DTO 134E9	34
A0A0U5G4R5	Cutinase	ASPCAL05624	*Aspergillus calidoustus*	39
A0A5M3ZEU8	Cutinase	ATEIFO6365_0012040500 ATETN484_0013041600	*Aspergillus terreus*	34
A0A2V5J3U4	Cutinase	BP00DRAFT_421233	*Aspergillus indologenus* CBS 114.80	20
A0A397GH94	Cutinase	CDV56_106452	*Aspergillus thermomutatus*	33
A0A8T8X123	Cutinase	BO86DRAFT_363433	*Aspergillus japonicus* CBS 114.51	24
A0A8H4MFB4	Cutinase	CNMCM6457_006370 CNMCM6805_002367	*Aspergillus fumigatiaffinis*	31

A0A1L9PSA9	Cutinase	ASPVEDRAFT_135708	*Aspergillus versicolor* CBS 583.65	34
A0A395HZB8	Cutinase	BO97DRAFT_424061	*Aspergillus homomorphus* (strain CBS 101889)	21
A0A3D8QAR6	Cutinase	DSM5745_11148	*Aspergillus mulundensis*	37
A0A8H6QN03	Cutinase	CNMCM7691_003472	*Aspergillus felis*	35
A0A5N6G083	Cutinase	BDW43DRAFT_299853	*Petromyces alliaceus (Aspergillus alliaceus)*	35
A0A5N7CLU8	Cutinase	BDV23DRAFT_179607	*Petromyces alliaceus (Aspergillus alliaceus)*	34
A0A8H5ZU22	Cutinase	ETB97_008594	*Aspergillus burnettii*	34
A0A8H6PWP0	Cutinase	CNMCM5623_007841	*Aspergillus felis*	33
A0A7R8AUQ3	Cutinase	APUU_80418A	*Aspergillus puulaauensis*	33
A0A1L9WYN5	Cutinase	ASPACDRAFT_77135	*Aspergillus aculeatus* (strain ATCC 16872 / CBS 172.66 / WB 5094)	20
I8TVI3	Cutinase	Ao3042_05584	*Aspergillus oryzae* (strain 3.042) (Yellow koji mold)	32
A0A319CRT5	cutinase	BO82DRAFT_302097	*Aspergillus uvarum* CBS 121591	20
A0A7R7X9D8	cutinase	APUU_10100A	*Aspergillus puulaauensis*	28
A0A5M3Z9Q0	Cutinase	ATEIFO6365_0009043300 ATETN484_0011042800	*Aspergillus terreus*	31
A0A3M7KGZ2	Cutinase	CA14_008605 F9C07_5814	*Aspergillus flavus*	32
A0A1S9DDD9	Cutinase	OAory_01092880	*Aspergillus oryzae* (Yellow koji mold)	32
A0A5N6GU43	Cutinase	BDV35DRAFT_381430	*Aspergillus flavus*	32
A0A7G5JIB6	Cutinase	G4B84_010849	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	32
A0A2I2GDH8	Cutinase	P170DRAFT_493134	*Aspergillus steynii* IBT 23096	35
A0A2P2H7M9	Cutinase	AFLA70_17g005581	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	32
A0A319DH01	cutinase	BO71DRAFT_168140	*Aspergillus ellipticus* CBS 707.79	32
A0A5N6HSU4	Cutinase	BDV32DRAFT_161169 BDV37DRAFT_286798	*Aspergillus pseudonomiae*	33
A0A5N6IVJ9	Cutinase	BDV30DRAFT_161452	*Aspergillus minisclerotigenes*	33

A0A3D8RKW1	Cutinase	DSM5745_07260	*Aspergillus mulundensis*	33
A0A1L9TEZ3	Cutinase	ASPSYDRAFT_90176	*Aspergillus sydowii* CBS 593.65	33
A0A2I2GLW0	Cutinase	P170DRAFT_396811	*Aspergillus steynii* IBT 23096	30
A0A5N7BSL4	Cutinase	BDV23DRAFT_176761	*Petromyces alliaceus (Aspergillus alliaceus)*	35
A0A8H6E4L3	Cutinase	ETB97_004682	*Aspergillus burnettii*	35
A0A5N6XC86	Cutinase	BDV39DRAFT_191631	*Aspergillus sergii*	32
A0A5N6FWJ5	Cutinase	BDW43DRAFT_311365	*Petromyces alliaceus (Aspergillus alliaceus)*	34
A0A5N6TT50	Cutinase	BDV25DRAFT_167948	*Aspergillus avenaceus*	30
A0A1L9PUL6	Cutinase	ASPVEDRAFT_44769	*Aspergillus versicolor* CBS 583.65	28
A0A319A0T6	cutinase	BO96DRAFT_401534	*Aspergillus lacticoffeatus* (strain CBS 101883)	31
A0A370BP75	cutinase	M747DRAFT_334854	*Aspergillus niger* ATCC 13496	31
A0A370P6W8	cutinase	M752DRAFT_309027	*Aspergillus phoenicis* ATCC 13157	31
A0A0F0I5Q9	Cutinase	P875_00042442	*Aspergillus parasiticus* (strain ATCC 56775 / NRRL 5862 / SRRC 143 / SU-1)	33
A0A2G7EMS2	Cutinase	AARAC_006644 BDV24DRAFT_153606	*Aspergillus arachidicola*	33
A0A5N6DBD4	Cutinase	BDV34DRAFT_228422	*Aspergillus parasiticus*	33
A0A5N6EY06	Cutinase	BDV33DRAFT_189903	*Aspergillus novoparasiticus*	33
A0A5N5WND0	Cutinase	BDV29DRAFT_63803	*Aspergillus leporis*	33
A0A1F7ZNN2	Cutinase	ABOM_010224	*Aspergillus bombycis*	32
A0A5N6W7N0	Cutinase	BDV41DRAFT_573371	*Aspergillus transmontanensis*	33
A0A5N6UIF6	Cutinase	BDV40DRAFT_22848	*Aspergillus tamarii*	33
A0A254UE39	cutinase	ATCC64974_87060 CAN33_0028750	*Aspergillus niger*	31
G3YBD6	cutinase	ASPNIDRAFT_178346	*Aspergillus niger* (strain ATCC 1015 / CBS 113.46 / FGSC A1144 / LSHB Ac4 / NCTC 3858a / NRRL 328 / USDA 3528.7)	31
A0A0F6TMI2	cutinase		*Aspergillus niger*	31
A0A3F3QCH7	cutinase	BDQ94DRAFT_184864	*Aspergillus welwitschiae*	31

A0A401KLM2	cutinase	AAWM_03026	*Aspergillus awamori* (Black koji mold)	31
A0A1L9T4G9	Cutinase	ASPSYDRAFT_497571	*Aspergillus sydowii* CBS 593.65	28
A0A5N6SNJ4	Cutinase	BDV38DRAFT_294944	*Aspergillus pseudotamarii*	33
A0A0L1JBR6	Cutinase	ANOM_002174	*Aspergillus nomiae* NRRL 13137	33
A0A5N7DK51	Cutinase	BDV32DRAFT_159450 BDV37DRAFT_291811	*Aspergillus pseudonomiae*	33
A0A5N7E6E9	Cutinase	BDV36DRAFT_303513	*Aspergillus pseudocaelatus*	33
A0A5N7A7I6	Cutinase	BDV27DRAFT_106441	*Aspergillus caelatus*	33
A0A317UNB9	cutinase	BO83DRAFT_350279	*Aspergillus eucalypticola* (strain CBS 122712 / IBT 29274)	32
A0A5N5XA21	Cutinase	BDV29DRAFT_189709	*Aspergillus leporis*	33
A0A0L1J803	Cutinase	ANOM_004397	*Aspergillus nomiae* NRRL 13137	32
A0A1L9U368	cutinase	ASPBRDRAFT_201334	*Aspergillus brasiliensis* (strain CBS 101740 / IMI 381727 / IBT 21946)	29
A0A319B002	cutinase	BO88DRAFT_395002	*Aspergillus vadensis* (strain CBS 113365 / IMI 142717 / IBT 24658)	30
A0A8G1RKL3	cutinase	BO72DRAFT_386881	*Aspergillus fijiensis* CBS 313.89	14
A0A2I1DBE8	Cutinase	P168DRAFT_324625	*Aspergillus campestris* IBT 28561	24
A0A1L9N071	cutinase	ASPTUDRAFT_30574	*Aspergillus tubingensis* (strain CBS 134.48)	30
A0A8H3XZK1	cutinase	AtWU_06368	*Aspergillus tubingensis*	30
A0A146F7K1	cutinase	ALUC_80022A RIB2604_01500270	*Aspergillus kawachii* (White koji mold) *(Aspergillus awamori var. kawachi)*	31
G7X9W2	cutinase	AKAW_02279	*Aspergillus kawachii (strain NBRC 4308) (White koji mold) (Aspergillus awamori var. kawachi)*	31
A0A8G1R1U9	cutinase	BO85DRAFT_514511	*Aspergillus piperis* CBS 112811	31
A0A318Z1U1	cutinase	BO87DRAFT_406722	*Aspergillus neoniger* (strain CBS 115656)	30
A0A5N7B504	Cutinase	BDV26DRAFT_293952	*Aspergillus bertholletiae*	34
A0A3M2T2T7	Cutinase	PHISP_05212	*Aspergillus* sp. HF37	27

A0A100IIR3	cutinase	ABL_04628	*Aspergillus niger*	29
A0A5N6XIV9	Cutinase	BDV39DRAFT_156890	*Aspergillus sergii*	34
A0A317V5S1	cutinase	BO70DRAFT_374354	*Aspergillus heteromorphus* CBS 117.55	30
A0A5N6YSB2	Cutinase	BDV24DRAFT_122259	*Aspergillus arachidicola*	35
A0A1M3T2V8	cutinase	ASPFODRAFT_200095	*Aspergillus luchuensis* (strain CBS 106.47)	31
A0A319DXE1	cutinase	BO78DRAFT_432820	*Aspergillus sclerotiicarbonarius* (strain CBS 121057 / IBT 28362)	31
A0A2V5IKA0	cutinase	BP00DRAFT_248697	*Aspergillus indologenus* CBS 114.80	33
A0A1F7ZZL4	Cutinase	ABOM_006753	*Aspergillus bombycis*	35
A0A5N6SIP8	Cutinase	BDV38DRAFT_296010	*Aspergillus pseudotamarii*	34
A0A318YZR5	cutinase	BP01DRAFT_308163	*Aspergillus saccharolyticus* JOP 1030-1	33
A0A2V5HDA3	cutinase	BO99DRAFT_471494	*Aspergillus violaceofuscus* (strain CBS 115571)	33
A0A8T8XBB5	cutinase	BO86DRAFT_406705	*Aspergillus japonicus* CBS 114.51	33
A0A5N6IYP7	Cutinase	BDV30DRAFT_240856	*Aspergillus minisclerotigenes*	34
A0A1S9DX15	Cutinase	OAory_01013580	*Aspergillus oryzae* (Yellow koji mold)	34
A0A2P2H724	Cutinase	AFLA70_51g004430	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	34
A0A364MGP4	Cutinase	BDV35DRAFT_161138 CA14_005054 F9C07_1000767	*Aspergillus flavus*	34
A0A5N6VJ71	Cutinase	BDV41DRAFT_568392	*Aspergillus transmontanensis*	34
A0A7G5ITT9	Cutinase	G4B84_002026	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	34
I8IKX6	Cutinase	Ao3042_03951	*Aspergillus oryzae* (strain 3.042) (Yellow koji mold)	34
A0A2I2FE37	Cutinase	BDW47DRAFT_117073	*Aspergillus candidus*	22
A0A5N6EKN1	Cutinase	BDV33DRAFT_233170	*Aspergillus novoparasiticus*	34

A0A2V5IL09	cutinase	BO99DRAFT_442309	*Aspergillus violaceofuscus* (strain CBS 115571)	11
A0A5N6UJ97	Cutinase	BDV40DRAFT_11719	*Aspergillus tamarii*	34
A0A5N7AB48	Cutinase	BDV27DRAFT_170168	*Aspergillus caelatus*	34
A0A2J5HD89	Cutinase	BDW42DRAFT_198138	*Aspergillus taichungensis*	23
A0A5N6DYL5	Cutinase	BDV34DRAFT_187451	*Aspergillus parasiticus*	34
A0A317X546	cutinase	BO94DRAFT_461645	*Aspergillus sclerotioniger* CBS 115572	31
A0A5N7ERJ7	Cutinase	BDV36DRAFT_305145	*Aspergillus pseudocaelatus*	34
A0A319CL39	cutinase	BO82DRAFT_132734	*Aspergillus uvarum* CBS 121591	34
A0A1L9X6A3	cutinase	ASPACDRAFT_75480	*Aspergillus aculeatus* (strain ATCC 16872 / CBS 172.66 / WB 5094)	34
A0A8H5ZVS9	Cutinase	ETB97_007077	*Aspergillus burnettii*	31
A0A395IA07	cutinase	BO97DRAFT_179619	*Aspergillus homomorphus* (strain CBS 101889)	34
A0A8G1W2X3	cutinase	BO72DRAFT_452	*Aspergillus fijiensis* CBS 313.89	34
A0A8H6UYW6	cutinase	CNMCM5793_003128 CNMCM6106_004216	*Aspergillus hiratsukae*	11
A0A318ZQ58	cutinase	BP01DRAFT_5084	*Aspergillus saccharolyticus* JOP 1030-1	10
A0A5N6U376	Cutinase	BDV25DRAFT_45229	*Aspergillus avenaceus*	32
A0A395H077	cutinase	BO80DRAFT_338094	*Aspergillus ibericus* CBS 121593	31
A0A1R3RXW8	cutinase	ASPCADRAFT_504731	*Aspergillus carbonarius* (strain ITEM 5010)	30
A0A7R7WJX2	cutinase	AKAW2_80022A	*Aspergillus kawachii* (White koji mold) *(Aspergillus awamori var. kawachi)*	10
A0A5N6WT77	Cutinase	BDV39DRAFT_195627	*Aspergillus sergii*	8
A0A2G7FJH2	Cutinase	AARAC_001660	*Aspergillus arachidicola*	5
A0A5N7BHQ0	Cutinase	BDV26DRAFT_95767	*Aspergillus bertholletiae*	2
Q5B2C1	Cutinase 1 (Ancut1) (Cutin hydrolase 1)	cut1 AN5309	*Emericella nidulans* (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) *(Aspergillus nidulans)*	43

P52956	Cutinase 1 (Cutin hydrolase 1) (L1)	cutL CutL1 AO090005000029	*Aspergillus oryzae* (strain ATCC 42149 / RIB 40) (Yellow koji mold)	34
Q5AVY9	Cutinase 2 (Ancut2) (Cutin hydrolase 2)	cut2 AN7541	*Emericella nidulans* (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) *(Aspergillus nidulans)*	36
Q5AX00	Cutinase 3 (Ancut3) (Cutin hydrolase 3)	cut3 AN7180	*Emericella nidulans* (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) *(Aspergillus nidulans)*	38
C8VJF5	Cutinase 4 (Ancut4)	cut4 ANIA_10346	*Emericella nidulans* (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) *(Aspergillus nidulans)*	8
A0A3M7JK39	Esterase	CA14_011952	*Aspergillus flavus*	1
A0A1F7ZYW7	Esterase	ABOM_007423	*Aspergillus bombycis*	1
A0A2G7G2E4	Esterase	AARAC_010280	*Aspergillus arachidicola*	1
A0A2I2FGC4	Esterase	BDW47DRAFT_116473	*Aspergillus candidus*	2
A0A2J5HQQ6	Esterase	BDW42DRAFT_195067	*Aspergillus taichungensis*	2
A0A2I1CZD4	Esterase	P168DRAFT_328088	*Aspergillus campestris* IBT 28561	1
A0A8E0R1U9	Esterase EstB	Aud_009318 IFM46972_07683	*Aspergillus udagawae*	2
A0A8H3S9B7	Esterase EstB	IFM51744_08255	*Aspergillus udagawae*	2
A0A8H3RXU8	Esterase EstB	IFM51744_04986	*Aspergillus udagawae*	2
A0A8H3PDH8	Esterase EstB	IFM46972_09273	*Aspergillus udagawae*	2
B8NB10	Esterase, putative	AFLA_043850 G4B84_010758	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	1
A2R2W3	Probable cutinase 1 (Cutin hydrolase 1)	An14g02170	*Aspergillus niger* (strain ATCC MYA-4892 / CBS 513.88 / FGSC A1513)	44

B8NCM8	Probable cutinase 1 (Cutin hydrolase 1)	AFLA_039350	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	43
B0XRY3	Probable cutinase 1 (Cutin hydrolase 1)	AFUB_025250	*Aspergillus fumigatus* (strain CBS 144.89 / FGSC A1163 / CEA10) *(Neosartorya fumigata)*	37
Q4X1N0	Probable cutinase 1 (Cutin hydrolase 1)	AFUA_2G09380	*Aspergillus fumigatus (*strain ATCC MYA-4609 / CBS 101355 / FGSC A1100 / Af293) *(Neosartorya fumigata)*	37
A1DGN0	Probable cutinase 1 (Cutin hydrolase 1)	NFIA_084890	*Neosartorya fischeri* (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) *(Aspergillus fischerianus)*	36
A1CSZ4	Probable cutinase 1 (Cutin hydrolase 1)	ACLA_081180	*Aspergillus clavatus* (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1 / QM 1276 / 107)	36
Q0CD01	Probable cutinase 1 (Cutin hydrolase 1)	ATEG_08433	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	35
B8MVS3	Probable cutinase 1 (Cutin hydrolase 1)	AFLA_072700	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	34
B0Y537	Probable cutinase 2 (Cutin hydrolase 2)	AFUB_071270	*Aspergillus fumigatus (*strain CBS 144.89 / FGSC A1163 / CEA10) *(Neosartorya fumigata)*	34
Q4WQV2	Probable cutinase 2 (Cutin hydrolase 2)	AFUA_4G14120	*Aspergillus fumigatus* (strain ATCC MYA-4609 / CBS 101355 / FGSC A1100 / Af293) *(Neosartorya fumigata)*	34

A1CVT3	Probable cutinase 2 (Cutin hydrolase 2)	NFIA_102190	*Neosartorya fischeri* (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) *(Aspergillus fischerianus)*	34
Q2TZY7	Probable cutinase 2 (Cutin hydrolase 2)	AO090011000665	*Aspergillus oryzae* (strain ATCC 42149 / RIB 40) (Yellow koji mold)	32
B8NBB2	Probable cutinase 2 (Cutin hydrolase 2)	AFLA_044870	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	32
A5ABE6	Probable cutinase 2 (Cutin hydrolase 2)	An11g00110	*Aspergillus niger* (strain ATCC MYA-4892 / CBS 513.88 / FGSC A1513)	31
Q0CES4	Probable cutinase 2 (Cutin hydrolase 2)	ATEG_07810	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	33
Q2U199	Probable cutinase 3 (Cutin hydrolase 3)	AO090011000113	*Aspergillus oryzae* (strain ATCC 42149 / RIB 40) (Yellow koji mold)	43
A1D9W1	Probable cutinase 3 (Cutin hydrolase 3)	NFIA_030250	*Neosartorya fischeri* (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) *(Aspergillus fischerianus)*	38
B0YEP5	Probable cutinase 3 (Cutin hydrolase 3)	AFUB_099910	*Aspergillus fumigatus* (strain CBS 144.89 / FGSC A1163 / CEA10) *(Neosartorya fumigata)*	36
Q4W9Z4	Probable cutinase 3 (Cutin hydrolase 3)	AFUA_4G03210	*Aspergillus fumigatus* (strain ATCC MYA-4609 / CBS 101355 / FGSC A1100 / Af293) *(Neosartorya fumigata)*	36
Q0CNE3	Probable cutinase 3 (Cutin hydrolase 3)	ATEG_04791	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	37

A1C9G0	Probable cutinase 3 (Cutin hydrolase 3)	ACLA_055320	*Aspergillus clavatus* (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1 / QM 1276 / 107)	33
Q0CW01	Probable cutinase 4 (Cutin hydrolase 4)	ATEG_02133	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	39
Q0CRP4	Probable cutinase 5 (Cutin hydrolase 5)	ATEG_03640	*Aspergillus terreus* (strain NIH 2624 / FGSC A1156)	36
A0A2P2HGU2	Putative esterase	AFLA70_104g002230	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	2
A0A7G5KHI7	Putative esterase	F9C07_5712	*Aspergillus flavus*	1
A0A7U2QT50	Putative transesterase	F9C07_6953	*Aspergillus flavus*	2
A0A7U2MVL1	Putative transesterase	F9C07_10476	*Aspergillus flavus*	2
A0A2P2H2C0	Putative transesterase (LovD)	AFLA70_189g002520	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	2
A0A370BF56	Transesterase	M747DRAFT_291061	*Aspergillus niger* ATCC 13496	2
A0A3M7JGM9	Transesterase	CA14_006281	*Aspergillus flavus*	2
A0A2P2GZT8	Transesterase	AFLA70_49g004131	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	2
A0A2G7FK89	Transesterase	AARAC_006061	*Aspergillus arachidicola*	1
A0A2G7FTA1	Transesterase (LovD)	AARAC_001835	*Aspergillus arachidicola*	2
A0A3M7JSE7	Transesterase (LovD)	CA14_001688	*Aspergillus flavus*	2
A0A0J5SF64	Transesterase (LovD)	Y699_08956	*Aspergillus fumigatus* Z5	2
A0A2G7G5L1	Transesterase (LovD)	AARAC_001565	*Aspergillus arachidicola*	2
A0A2P2HSS5	Transesterase (LovD)	AFLA70_70g003011	*Aspergillus flavus* (strain ATCC MYA-384 / AF70)	2
B8NEQ7	Transesterase (LovD), putative	AFLA_063440	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	2
B8NR26	Transesterase (LovD), putative	AFLA_004100	*Aspergillus flavus* (strain ATCC 200026 / FGSC A1120 / IAM 13836 / NRRL 3357 / JCM 12722 / SRRC 167)	2