Biodegradation of Polyhydroxybutyrate by Bacterial Strains, Native Extracellular PHB Depolymerases, and Structural Variants

by

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Abstract

Thanks to its biodegradability and biocompatibility, among other properties, the biodegradable polymer polyhydroxybutyrate (PHB) has the potential to become an extensively used plastic in the production of a variety of products, from polymer films and everyday objects to specialized devices like biosensors. Extracellular PHB depolymerases (PhaZs) — enzymes responsible for the biodegradation of this polymer — play a crucial role in understanding the life cycle of PHB and developing new PHB-based applications. Even though these enzymes have been investigated since the 1960s, new discoveries in this field continue to improve knowledge of PhaZs, their mechanisms and their properties. In addition, the tools provided by molecular and synthetic biology open the door for new applications and new enzymatic features. This dissertation presents advances in the understanding of PhaZs and their degradation of PHB that can lead to the development of PHB and PhaZs-based applications.

Rigorous comparisons of PHB degradation by nine bacterial strains with either demonstrated or predicted PhaZ activity were performed through bioinformatics and experimental approaches. This enabled direct assessments, which were previously not possible from literature as correlations between different studies conducted under different conditions are, at the very least, very difficult; and demonstrated that at least one predicted strain could perform PHB degradation. The observations obtained can provide a starting point for applications such as PHB recycling.

Next, a rationalized method to produce recombinant PhaZs was presented. This approach was demonstrated with five purified PhaZs (and four PhaZ variants in a

subsequent study). Through a simple and rapid PHB plate-based method, the activity of five recombinant PhaZs was rapidly compared at different temperatures and pH values. These methods can be used to produce and test PhaZs of native or synthetic origin.

The activity of a PhaZ from the marine strain *Marinobacter algicola* DG893 ($PhaZ_{Mal}$) was then experimentally validated and characterized, adding a new entry to the PhaZs found in literature. This enzyme is of interest as it is one of the few known PhaZs from marine bacteria, and because marine environments are specifically under threat due to the plastic accumulation crisis. In addition, comparisons were established with the well characterized PhaZ from the soil originating bacterium *Comamonas testosteroni* 31A ($PhaZ_{Cte}$).

$PhaZ_{Cte}$ was the focus of the final study presented in this dissertation due to its high activity and other attractive features. The structure of $PhaZ_{Cte}$ was modified through the removal or inclusion of domains and the fusion with protein sequences showing no affinity to PHB. This allowed the determination of the contribution of the domains to degradation, and of the robustness of this enzyme and its variants under several storage conditions. In addition, the demonstration that activity was retained when the enzyme is fused to the small outer capsid protein (Soc) from bacteriophage T4, shows potential for the integration of this enzyme in a bacteriophage expression system and in enzyme-responsive biosensors.

Preface

Part of this thesis has been previously published and some chapters will be submitted for publication. Additional acknowledgements are provided at the end of each specific chapter.

Chapter 3 was published as Martínez-Tobón D. I., Gul M., Elias A. L., and Sauvageau D. (2018) "Polyhydroxybutyrate (PHB) biodegradation using bacterial strains with demonstrated and predicted PHB depolymerase activity" in Applied Microbiology and Biotechnology 102, 8049–8067. doi: 10.1007/s00253-018-9153-8. As the primary author, I was responsible for conceptualization, bioinformatics analysis, designing, performing and analyzing experiments, and writing the manuscript. Maryam Gul was an undergraduate student who participated in PHB film fabrication, strains growth, and bacterial PHB degradation experiments under my co-supervision. Dr. Dominic Sauvageau and Dr. Anastasia Elias were the supervisory authors who contributed from conceptualization, data interpretation, and advice, to manuscript composition.

Chapter 4 and Chapter 5 will be submitted for publication as Martínez-Tobón D. I., Waters B., Elias A. L., and Sauvageau D. "Streamlined production, purification, and comparison of recombinant extracellular polyhydroxybutyrate depolymerases" and Martínez-Tobón D. I., Waters B., Elias A. L., and Sauvageau D. "A novel polyhydroxybutyrate depolymerase from the marine bacterium *Marinobacter algicola* DG893", respectively. As the primary author in both chapters, I was responsible for conceptualization, bioinformatics analysis, designing, performing and analyzing experiments, and writing the manuscripts. Brennan Waters was involved in the culture of

strains with predicted PhaZ activity with modified media and the production of recombinant PhaZ$_{Mal}$ from primer design to initial purification under my co-supervision. Dr. Dominic Sauvageau and Dr. Anastasia Elias were the supervisory authors who contributed from conceptualization, data interpretation, and advice, to manuscript composition.

Chapter 6 will be submitted for publication as Martínez-Tobón D. I., Harrison M., Storms Z., Elias A. L., and Sauvageau D. "Structural variants of extracellular polyhydroxybutyrate depolymerase from *Comamonas testosteroni* impact degradation rates". As the primary author, I was responsible for conceptualization, designing, performing and analyzing experiments, and writing the manuscript. Melissa Harrison and Dr. Zachary Storms designed and produced the plasmid constructs of the C, CL, SCLB, and CLS variants, and proofread the manuscript. Dr. Dominic Sauvageau and Dr. Anastasia Elias were the supervisory authors who contributed from conceptualization, data interpretation, and advice, to manuscript composition.

This thesis is dedicated to my mother, father, and sister,

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List of Abbreviations

3HB	3-hydroxybutyric acid, 3-hydroxybutyrate
AFM	Atomic force microscopy
ANOVA	Analysis of variance
Ap	Ampicillin
Asn	Asparagine
Asp	Aspartic acid (also D)
BLAST	Basic Local Alignment Search Tool
BLASTp	Protein BLAST
BPB	Bromophenacyl bromide, 2,4′-dibromoacetophenone
C	PhaZ$_{Cte}$ variant with catalytic domain only
Cat	PhaZ catalytic domain (also C in Chapter 6)
CL	PhaZ$_{Cte}$ variant without SBD
CLB	Wild-type PhaZ$_{Cte}$
CLS	PhaZ$_{Cte}$ variant with Soc replacing the SBD
CoA	Coenzyme A
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
Cys	Cysteine
DEAE	Diethylaminoethyl
DPN	Diphosphopyridine nucleotide, nicotinamide adenine dinucleotide (also NAD)
DSMZ	German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
DTE	Dithioerythritol
DTT	Dithiothreitol
EC	Enzyme Commission
e-dPHAscl	Extracellular - denatured short chain length PHAs
EIC	Extracted ion chromatograms
Fn3	Fibronectin type III
GC	Gas chromatography
gDNA	Genomic DNA
Gly	Glycine
GO	Gene ontology
GPC	Gel permeation chromatography
GST	Glutathione-S-transferase

HA	Hydroxyalkanoate
His	Histidine (also H)
H-NMR	Proton nuclear magnetic resonance
HPLC	High-performance liquid chromatography
IF	Insoluble fraction
i-PhaZ	Intracellular PhaZ
IPTG	Isopropyl-β-d-thiogalactopyranoside
JCM	Japan Collection of Microorganisms
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
link	PhaZ linker domain (also L in Chapter 6)
M	Methionine (also m)
MAFFT	Multiple Alignment using Fast Fourier Transform
MalE	Maltose-binding protein from *Escherichia coli*
mCLB	Wild-type PhaZ$_{Cte}$ with an additional methionine
Mn	Number-average molecular weight
MS	Mass spectrometry
Mw	Molecular weight, weight-average molecular weight (GPC data)
Mw/Mn	Polydispersity index
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OD	Optical density
OD_{210}	Optical density measured at wavelength of 210 nm
OD_{600}	Optical density measured at wavelength of 600 nm
P(3HB-co-4HB)	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
P(3HHx-co-3HO)	Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)
P(4HB)	Poly(4-hydroxybutyrate)
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PET	Poly(ethylene terephthalate)
PHA	Polyhydroxyalkanoate
PHA-DED	PHA Depolymerase Engineering Database
PhaZ	PHA and PHB depolymerases (general), Extracellular PHB depolymerase (specified within the chapters when the term applies to this specific concept)
PhaZ$_{Csp}$	PhaZ from *Cupriavidus* sp.
PhaZ$_{Cte}$	PhaZ from *Comamonas testosteroni* 31A
PhaZ$_{Mal}$	PhaZ from *Marinobacter algicola* DG893
PhaZ$_{Msp}$	PhaZ from *Marinobacter* sp. NK-1

PhaZ$_{Pfu}$	PhaZ from *Penicillium funiculosum*
PhaZ$_{Pst}$	PhaZ from *Pseudomonas stutzeri*
PhaZ$_{Rpi}$T1	PhaZ from *Ralstonia pickettii* T1
PhaZ$_{Rsp}$	PhaZ from *Ralstonia* sp.
PHB	Polyhydroxybutyrate, poly(3-hydroxybutyrate) (also P(3HB))
PHBHHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (also PHBHV and P(3HB-co-3HV))
PHV	Polyhydroxyvalerate, poly(3-hydroxyvalerate) (also P(3HV))
pI	Isoelectric point
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
PNPB	*p*-nitrophenyl butyrate
PP	Polypropylene
QCM	Quartz crystal microbalance
RI	Refractive index
rPhaZ	Recombinant PhaZ
RT	Room temperature
SBD	PhaZ substrate binding domain (also B in Chapter 6)
SCL	Short chain length
SCLB	PhaZ$_{Cte}$ variant of wild-type enzyme with N-terminal Soc
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
Ser	Serine (also S in Chapter 2 and Chapter 3)
SF	Soluble fraction
SIB	Swiss Institute of Bioinformatics
Soc	Small outer capsid protein of bacteriophage T4 (also S in Chapter 6)
SOC	Super Optimal broth with Catabolite repression (culture medium)
SP	PhaZ signal peptide
T_c	Cold-crystallization peak temperature
T_g	Glass transition temperature
T_m	Melting temperature
T_{onset}	Onset cold-crystallization temperature
TrxA	Thioredoxin
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet

1. Introduction

1.1. Motivation and context

The everyday nature of plastic usage is undeniable. Given the development of processes that allow low-cost mass production, and their beneficial characteristics — lightweight, durability, flexibility, and easiness to process — plastics are used in numerous applications, packaging being the most common one. Most widely used plastics are made of synthetic polymers such as polyethylene, polypropylene, polystyrene, nylon and polyurethane. Currently, most synthetic polymers are produced from non-renewable petrochemical resources and are not degradable in the environment, leading to an immense problem facing our society: plastic waste accumulation.

An analysis of plastics manufacture and accumulation published in 2017 estimated that, at that point, 8,300 million metric tons of virgin plastics had been produced, and that by 2015 6,300 million metric tons of plastic waste had been generated — with 79% ending up in landfills or the environment, 12% incinerated, and only 9% recycled [1]. Even an increase in recycling would only be a partial solution, as chemical recycling is expensive and still under development. Moreover, mechanical recycling — the method typically implemented — is labour extensive, leads to lower quality plastics, and is limited to certain applications [2-4]. On the other hand, thermal destruction entails significant health and environmental impacts, even if some energy can potentially be recovered (depending on the process) [2,4].

This uncontrolled generation of plastic waste and its restricted management are causing great concerns. Beyond the environmental impact of large scale objects, the accumulating evidence that microplastics and their associated chemicals accumulate in the food chain, drinking water, and even the air we breathe, as well as the potential health effects of this exposure, is, to many, alarming [5-9]. Microplastics have even been detected in human stool in a preliminary study, where 8/8 samples analyzed showed the presence of up to 9 types of plastics [10,11]. Therefore, efforts are underway to reduce the amount of plastic waste produced; mostly through personal actions towards the reduction of plastic consumption and government led initiatives like bans on the use of microbeads or single-use plastic bags, for instance [12]. However, since the effects of those efforts are far exceeded by the rate at which plastic contamination is generated, and considering the needs for plastic materials in society, further strategies need to be put in motion.

A viable alternative to the use of synthetic polymers is the use of their biodegradable counterparts, which hold great potential for a wide range of applications, including packaging [13] and biomedical uses [14]. Biodegradation is broadly defined as degradation resulting from the activity of biological agents, like microorganisms and/or enzymes [15-17]. It should be considered that other conditions related to the environment, such as simple hydrolysis, contribute to this degradation and occur at the same time or can even trigger the biological degradation process [15]. Moreover, the 1992 international workshop on biodegradability (Annapolis, MD, USA) postulated that a biodegradable material must be associated to a specific means of disposal (compost, sewage treatment, denitrification, or anaerobic sludge treatment), with a degradation rate suitable for that method to control accumulation, that the degradation does not have a negative impact on

the process or end product, and that the material degrades into safe compounds like carbon dioxide, water, and minerals in the case of aerobic degradation [15].

There is a wide offer of biodegradable polymers, each with advantages and disadvantages, but polylactides (lactic acid-based polymers) and polyhydroxyalkanoates (PHAs) can be found among the most attractive options [2]. While polylactic acid (PLA) — obtained from the esterification of lactic acid produced by fermentation (although it can also be synthetized from oil) — is currently the most common type of biodegradable plastic due to large market availability and relatively low price [18]. Its biodegradation can only take place after an initial abiotic hydrolytic degradation step under specific temperature and moisture conditions that later allow microorganism attack [19] — in other words, under controlled industrial composting conditions so that it can be degraded within an acceptable time frame [18-20]. In addition, few studies have focused on enzymatic degradation of PLA catalyzed by proteinase K, serine proteases, lipases, cutinase-like enzymes, and a couple of PLA depolymerases, as reviewed by Tokiwa and Calabia [21], and Karamanlioglu et al. [22].

Another promising class of biopolyesters are PHAs — poly(3-hydroxybutyrate) (PHB) being its most abundant form [23]. These biopolymers of microbial origin are not prone to rapid abiotic hydrolysis (an undesired characteristic during processing, material storage or long-term applications [19,24]), and possess a diverse range of properties that are amenable to applications like packaging, coatings, and medical devices [2]. They originate from renewable resources and can be completely biodegraded into products innocuous to the environment through microbial and enzymatic action [2,25] — potentially, even under typical backyard conditions [20]. They thus represent promising,

sustainable alternatives to the growing problem of environmental plastics accumulation. Unlike PLA, the enzymes involved in the degradation of PHAs — namely PHA depolymerases (PhaZs) — have been broadly investigated [21]. However, efforts to expand the knowledge on PHA environmental degradation, the microorganisms mediating their degradation in diverse environments, and the mechanisms, characterization, and production of known and novel PhaZs must be continued as it is crucial to understand, enhance, and possibly control the degradation processes of the biopolymers intended to help solve the plastics waste issue. This knowledge also serves to develop innovative biopolymer-based biotechnologies in other spheres, and to potentially design novel PhaZ variants that can degrade biopolymers under particular expression or environmental conditions.

1.2. Scope of the thesis

The overall objective of this thesis project was to develop new knowledge on the enzymatic degradation of PHB and of extracellular PHB depolymerases (also denominated PhaZs) to enable future advances in PHB-based products and processes. Since PHB is a natural biopolymer synthesized by many microorganisms and part of many ecosystems, there is a large pool of unstudied PhaZs with potentially valuable characteristics to better understand the mechanisms of PHB degradation. Furthermore, these could be used to develop tailored processes for PHB recycling or removal, and even lead to engineering PhaZs that act faster, under a broader range of conditions to be employed in new processes and biotechnological applications. An example could be PHB-based biosensors that display a conformational change upon the action of a PhaZ for detection of pathogens in

food [26], or of a condition or analyte of interest in environmental, industrial, or health related processes [27].

The specific aims of the thesis were: 1) to compare PHB degradation by bacterial strains with either demonstrated or predicted extracellular PhaZ activity through bioinformatics and experimental approaches; 2) to develop a method to produce heterologous PhaZs to study their behaviour with PHB; 3) to characterize the newly isolated PhaZ from the strain *Marinobacter algicola* DG893 ($PhaZ_{Mal}$); and finally 4) to modify the structure of PhaZ from *Comamonas testosteroni* 31A ($PhaZ_{Cte}$) to determine how structural variants affect the kinetics and stability of the enzyme.

1.3. Thesis structure

Chapter 2 provides a review of biodegradable polymers and PHAs with emphasis on PHB. This includes a description of properties and the different degradation mechanisms of PHB, focusing on biodegradation by extracellular PhaZs, for which an up-to-date survey is presented. The review also gives a description of methods employed for recombinant production of PhaZs, assays used to measure their activity, and a brief description of PHB-PhaZs technologies.

In Chapter 3 the performance of several bacterial strains cultured under the same conditions with PHB film as the only carbon source is compared. The strains used in the study were selected based on either their previously demonstrated capacity to degrade PHB or the fact that their genomes contained a sequence predicting the presence of extracellular PhaZ functionality. Few if any studies presented in the literature compare PHB-degrading strains performing under the same set of conditions, as they only focus on degradation by

a single strain or enzyme; as an initial step, this work contributes to closing this gap. In addition, of the strains with predicted PhaZ activity, which were all isolated from marine environments, only *M. algicola* was able to degrade PHB films, validating for the first time its capacity to use PHB. A bioinformatics analysis of the protein structures of the PhaZs from all strains investigated was performed to further shed light on the causes and differences in biodegradation (or lack therefore) in the different strains.

In Chapter 4, a method to produce recombinant PhaZs is described to produce five heterologous PhaZs. The activity of the resulting isolated enzymes was compared under different pH and temperature conditions through a simple method based on agar plates containing PHB particles in suspension. This PhaZ production method sets the basis for the studies found in Chapter 5 and Chapter 6.

In Chapter 5, the focus moved to the new enzyme from *M. algicola* ($PhaZ_{Mal}$) which was identified in Chapter 3. The enzyme was characterized, and its activity assessed under a wide range of conditions. The optimal pH and temperature conditions were thus determined, and its degradation mechanism was defined. A study of medium components showed that the presence of $CaCl_2$ and NaCl aided in the degradation process, suggesting a possible need for metal ions for improvement of activity.

In Chapter 6, structural variants of $PhaZ_{Cte}$ were designed and produced to investigate how the removal of domains and the addition/replacement of a small decorating protein — Soc, the small outer capsid protein of bacteriophage T4 — impacted the kinetics and stability of $PhaZ_{Cte}$. The degradation activity of the variants was compared and helped decouple the contribution of the substrate binding, linker and catalytic domains of the enzyme.

This thesis concludes with Chapter 7 which summarizes the findings and contributions made from this work, as well as the conclusions that can be drawn from it. A description of the future directions that can be taken from the results of this project is also presented.

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2. Literature Review

2.1. Biodegradable polymers

Biodegradability is the capacity of organic materials (like polymers) to degrade due to biological activity — mainly enzymatic action of microorganisms — into products like carbon dioxide or methane (under aerobic and anaerobic conditions, respectively), new biomass, and water [1,2]. There are several types of biodegradable polymers and multiple ways to classify them, but based on their synthesis process, they can originate from plant or animal biomass (starch, cellulose, chitosan, proteins, among others), from microbial production (polyhydroxyalkanoates (PHAs), like poly(3-hydroxybutyrate) (PHB)), conventional synthesis with monomers from renewable resources (polylactides, like polylactic acid (PLA)), and from petrochemical products (polycaprolactone (PCL), polyvinyl alcohol, and other aliphatic or aromatic polyesters and co-polyesters) [1,3]. This last category poses a disadvantage in the sense that their source material is non-renewable. In this context, there is a broader classification for the word "biopolymers" (also called bioplastics), which can be defined by their source and/or biodegradability: 1. biopolymers originating from renewable raw materials (bio-based) that are biodegradable (broadly exemplified above), 2. bio-based biopolymers that are not biodegradable (e.g. biopolyethylene produced from bioethanol, or rubber), and 3. petrochemical-based biopolymers which are biodegradable [4].

Some of the bio-based polymer alternatives are limited to specific applications, based on their properties and processability. For example, PLA and PHB, which have

higher melting points than starch, and cellulose can only be processed through injection moulding, extrusion, and blowing, while PLA can also be processed through fibre spinning and thermo forming [3]. On the other hand, extrusion blowing and extrusion casting can be used to process a PHA like poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).

PLA is currently the most widely used biopolymer due to its comparatively low price and its large-scale commercial availability thanks to enterprises like NatureWorks, an affiliate of Cargill [1,3,5,6]. For its production, the monomer (lactic acid) can be produced through chemical synthesis — typically, petroleum resources are hydrolyzed, esterified, and purified into racemic DL-lactic acid (with acetaldehyde and lactonitrile as intermediates) — or fermentation — in which carbohydrates, usually agro-based, are converted to lactic acid by lactic acid bacteria or fungi [7]. The polymer can then be synthesized through polycondensation or, commercially, through ring-opening polymerization [7].

Abiotic hydrolysis is the main mechanism of PLA degradation, and biodegradation in compost seems to be possible under appropriate conditions in industrial units (above 50 °C, at high humidity) [1]. PLA can be hydrolyzed by enzymes like proteinase K and pronase, but these are unable to diffuse through the crystalline regions of the polymer, limiting biodegradation rates [7]. Moreover, preliminary abiotic hydrolysis of PLA is typically required for microorganisms to utilize the oligomers or lower molecular weight (Mw) components that diffuse to the surface of the bulk polymer [8]. After biodegradation, PLA turns into carbon dioxide, water, and humus [7].

PLA biodegradation is an example illustrating the complexity of biodegradation processes. Of course, this depends on the chemistry of the material, its raw components,

the structure of the final product, and the degradation environment and conditions it faces — such as presence of microorganisms, availability of oxygen, amount of available water, temperature, pH, and electrolytes present [2,9]. Some biodegradable polymers can biodegrade in a few weeks, while others can take months under the same environmental conditions or might only degrade under different conditions [2]. For instance, some compostable materials can biodegrade in simple soils while others cannot [2]. Another example comes from a one year comparative degradation study in artificial seawater and freshwater under controlled conditions (thermostatic chamber at 25 °C, cycles of 16 h fluorescent light and 8 h dark) of films of the biodegradable polymers poly(lactic-co-glycolic acid) (PLGA), PCL, PLA, PHB, Ecoflex (commercial compostable fossil-based polymer from BASF), and the non-degradable polymer poly(ethylene terephthalate) (PET) [10]. In both media tested, only PLGA degraded completely — after approximately 270 days through a bulk degradation mechanism — and PHB lost approximately 8% mass at the end of the study through a surface degradation mechanism [10]. None of the other polymers degraded significantly [10]. This demonstrates how important it is to identify the conditions under which biopolymers biodegrade, especially in consideration of their intended use. The following sections focus on PHAs, and mainly PHB, the biopolymer selected for this investigation, based on their renewable origin, their attractive properties making them suitable for diverse applications, their innocuous degradation products, but above all their significant interactions with a wide array of enzymes involved in their degradation: PHA and PHB depolymerases (PhaZs).

2.2. Polyhydroxyalkanoates

PHAs are a class of natural, biodegradable, and biocompatible polyesters, composed of repeated chains of hydroxyalkanoate(s) (HA) monomers, with thermoplastic and even piezoelectric, antioxidant, and tunable optical properties [5,11-15]. They are produced by many microorganisms as insoluble intracellular granules for carbon and energy storage during unbalanced growth [12,16]. The discovery of PHAs was first reported back in 1926, when Maurice Lemoigne discovered PHB granules — as well as the constituents of the polymer, 3-hydroxybutyrate (3HB), also known as 3-hydroxybutyric acid — in the strain *Bacillus megaterium* ([17] as reviewed in [5,15,16]). Over the following decades, other producing strains, homopolymers, copolymers, and their building blocks have been described. By the year 2004, more than 300 PHA-producing microbial strains and more than 100 PHAs had been identified [5], and by 2015 more than 200 different HAs had been reported [15]. It should be noted however, that only a few of these monomers can be naturally synthesized by bacteria; most of them can only be synthesized and accumulated if the cells are provided specific precursors or structurally related carbon sources, under specific culture conditions (examples include 4-hydroxybutyrate, 4-hydroxyvalerate, 4-hydroxyhexanoate, 5-hydroxyhexanoate, 4-hydroxyheptanoate, and 4-hydroxyoctanoate) [18]. However, this also shows that the pathways and enzymes in charge of synthesizing PHAs (like PHA synthase) are quite adaptable [18].

PHAs are classified according to the number of carbons of their monomer as short chain length (SCL) (3 to 5 carbon atoms), medium chain length (6 to 14 carbon atoms), and long chain length (more than 14 carbon atoms, not widely studied) [5,15,16]. In biosynthesized PHAs, the monomer units are always in the R-configuration [5,19,20].

Figure 2.1 shows a generalization of the chemical structure of PHAs [5,20,21] and some examples of PHAs are listed in Table 2.1 [20,22,23].

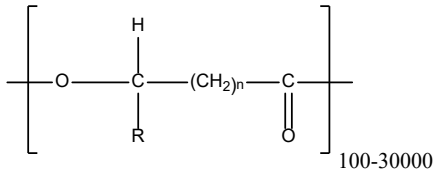

Figure 2.1 General chemical structure of PHAs.
R: side group, n = 1–4 [5,20,21].

Table 2.1 PHAs examples.
R: side group, n = 1–4 (See Figure 2.1) [20-23].

Homopolymer examples		
Chemical structure components	**Chemical name**	**Abbreviation(s)**
R = hydrogen, n = 1	Poly(3-hydroxypropionate)	P(3HP)
R = methyl, n = 1	Poly(3-hydroxybutyrate)	PHB, P(3HB)
R = ethyl, n = 1	Poly(3-hydroxyvalerate)	PHV, P(3HV)
R = propyl, n = 1	Poly(3-hydroxyhexanoate)	PHHx, P(3HHx)
R = butyl, n = 1	Poly(3-hydroxyheptanoate)	PHHp, P(3HH)
R = pentyl, n = 1	Poly(3-hydroxyoctanoate)	P(3HO)
R = nonyl, n = 1	Poly(3-hydroxydodecanoate)	PHDD, P(3HDD)
R = hydrogen, n = 2	Poly(4-hydroxybutyrate)	P(4HB)
R = methyl, n = 2	Poly(4-hydroxyvalerate)	P(4HV)
R = hydrogen, n = 3	Poly(5-hydroxyvalerate)	P(5HV)
R = methyl, n = 3	Poly(5-hydroxyhexanoate)	P(5HHx)
R = hexyl, n = 4	Poly(6-hydroxydodecanoate)	P(6HDD)
Copolymer examples		
Chemical name		**Abbreviation(s)**
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)		PHBV, PHBHV, P(3HB-co-3HV)
Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)		P(3HB-co-4HB)
Poly(3-hydroxyoctanoate-co-hydroxyhexanoate)		PHOHHx
Poly(3-hydroxybutyrate-co-3-hydroxyoctanoate)		PHBHO, PHBO
Poly(3-hydroxybutyrate-co-3-hydroxydecanoate)		PHBHD, PHBD
Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)		P(3HHx-co-3HO)

Many PHAs are hydrophobic, with their Mw ranging from several hundreds to a million Da. Many of their physical and chemical characteristics depend on their monomer content, which is affected by production conditions like microorganism type, media, fermentation conditions and mode, and even the recovery method [5,15,24]. Many PHAs show low permeability to water and oxygen, an important advantage in many packaging applications [25]. In some cases, their properties can be modified to make them more suitable for applications. For example, their hydrophobicity can be reduced through functionalization with hydroxyl, amine, or carboxylic groups or through graft copolymerization with hydrophilic components to facilitate drug delivery [23]. During biodegradation, PHAs are eventually converted to energy, carbon dioxide and water under aerobic conditions, or to energy, methane and water under anaerobic conditions [5,13,15].

Due to all these attractive characteristics, and since they have physicochemical properties similar to synthetic polymers such as polypropylene (PP) or polyethylene [5], PHAs have drawn significant commercial interest as potential replacements for many petrochemically-derived polymers. In addition, given the wide range of HAs [15] and available PHAs [5], there is an array of material properties to choose from for different applications. A couple of examples include P(4HB) which, based on its elasticity, Mw, absorbability, and biocompatibility (4HB is a common metabolite found in humans), has been successfully used in medical applications such as tissue engineering scaffolds or sutures [15,26], and PHBV which has been used in agricultural applications such as controlled release of pesticide and herbicide with minimal impact on the surrounding environment [15,27,28].

Large-scale production and research for commercial purposes has been mostly done with PHB, PHBV, P(4HB), P(3HB-co-4HB), P(3HHx-co-3HO), and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) [5,29,30]. Of these, PHB and PHBV have the largest commercial production, with a few hundred tons per year [31]. Production strategies include fed-batch growth of a few known PHA-producing microorganisms followed by accumulation of PHA under limitation conditions [5]. The companies that have ventured into PHAs production include American companies such as Metabolix (which has produced PHAs using bacterial strains and have conducted research efforts to produce PHB in switchgrass plants [32]), Danimer Scientific (previously Meridian, producer of Nodax a family of biopolymers based on PHBHHx and other PHAs [25,30]) , and Newlight Technologies, PHB Industrial S.A. in Brazil, Biomer in Germany, Goodfellow in the UK, NaturePlast in France, Bio-on in Italy, Kaneka Corporation in Japan, and Tianjin Green Bioscience (P(3HB-co-4HB) production [30]), Ecomann, and Tianan (largest world supplier of PHBV as of 2014 with 1000 tons/year [30]) in China [25,30,33-35]. In addition to these, smaller companies such as Mango Materials [36], TerraVerdae Bioworks, and others have worked on the development of methane to PHB processes. As the PHAs industry is under development, some of these companies have ceased to exist or their intellectual property has been transferred to other players — an example is Metabolix, now Yield10 Bioscience, which sold its intellectual property to the South Korean firm CJ CheilJedang Corp in 2016 to focus on the improvement of crop plants metabolism [37-39].

Since PHB is the most abundant, commonly used, and fully characterized PHA [5,13,33], it was selected for this work. The following sections and chapters are focused on this biopolymer and its degradation.

2.3. Polyhydroxybutyrate

PHB (linear formula: $[COCH_2CH(CH_3)O]_n$ [5]) is mostly produced industrially from sugars [30,40], but it has also been produced from methanol, ethanol, acetate, and even molasses, whey cheese, and hemicellulose hydrolysate [5,41]. PHB contents have been reported to reach 25–88% of dry cell weights [5]. Some examples of bacterial strains that have been used for PHB production include *Alcaligenes latus*, *Azotobacter vinelandii*, *Azotobactor chroococcum*, *Methylobacterium organophilum*, *Ralstonia eutropha* and other strains of the *Cupriavidus* genus [33], and recombinant strains of *Escherichia coli* and *Klebsiella aerogenes* [5,41]. Of these, *R eutropha*, *A. latus* and recombinant *E. coli* are the main strains for industrial-scale production — selected for high PHB production efficiency and use of carbon sources like glucose and ethanol [30,33].

2.3.1. Biosynthesis and crystallization of PHB

Three main enzymatic reactions are specifically involved in the biosynthesis of PHB: first, two Acetyl-coenzyme A (CoA) molecules are condensed into acetoacetyl-CoA by β-ketothiolase; second, reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA dehydrogenase reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA monomers, and third, the monomers are polymerized into PHB by PHB synthase [5,42-44]. PHB (as well as other PHAs) accumulates in the cell as submicron-sized granules [5,12,18]. The size of the granules, combined with a functional surface layer composed of proteins [5,12,18,45], help maintain PHB in an amorphous state with very slow crystallization rates, given that there are no nucleation sites due to the lack of impurities [5,46]. Once the granules are damaged or extracted (referred to as denatured

PHB), PHB starts crystallizing, becoming a semi-crystalline polymer at temperatures above its glass transition temperature (T_g), and stabilizing at approximately 60–90% crystallinity [5,47]. From differential scanning calorimetry thermograms of quenched (amorphous) samples, the onset cold-crystallization temperature (T_{onset}) of PHB was determined to be around 38 °C and its cold-crystallization peak temperature (T_c) is about 47 °C [48]. Crystallization occurs by the formation of spherulites of lamellar crystals (monolamellar for single crystals and multi-oriented for bulk materials like films) [5].

2.3.2. Properties of PHB

Among other properties, PHB is a stereoregular — isotactic with monomers in the (R)-configuration — optically active polymer, [5,18], with typical density values in its crystalline and amorphous forms of 1.26, and 1.18 g/cm^3, respectively [49]. Its melting temperature (T_m) is approximately 180 °C [18], although it can vary between 165 and 180 °C depending on its crystallization temperature [50]. Its T_g has been reported to be between 2.5 °C [48] and 4 °C [18]. As with other PHAs, the Mw of PHB ranges from several hundred to several million Da (depending on strain, fermentation and extraction conditions), with polydispersity index (Mw/Mn) values in the range of 2.3–3.2 [5,51].

Biocompatibility is an important characteristic of PHB [5]; the monomeric component (R-3HB) is a common metabolite found at 0.03–0.1 mg/ml in human blood ([52] and [53], as cited in [5]), and its oral toxicity is insignificant with lethal dose LD_{50} > 5000 mg/kg [47]. In addition, PHB has better gas barrier properties and is more resistant to damage by ultraviolet (UV) radiation than PP and polystyrene [5,54,55]. In addition, after reaching its crystallization thermodynamic end point at room temperature (no free

amorphous mass left to change since Tg is near 0 °C), PHB remains very stable, seeing little to no change in mechanical properties [55]. Although the mechanical properties of PHB are dependent on many factors, reported values for Young's modulus are 3.5 GPa and 43 MPa for tensile strength, which are close to the values of isotactic PP [18]. However, one of the disadvantages of PHB is that its elongation at break is very low: approximately 5% compared to 400% for PP, rendering it stiff and brittle [5,18]. This is likely due to its high crystallinity as the formation of cracks in spherulites is often associated with embrittlement [18].

2.3.3. Efforts to improve PHB properties

As PHB is a cost competitive PHA and has many favourable properties, there have been efforts to overcome the stiffness and brittleness issues through processing [18,29,47]. Examples include the preparation of uniaxially oriented films through polymer drawing techniques from amorphous PHB [56], and the use of ultra-high-Mw PHB with a two-step drawing procedure [57]. This improved its mechanical properties (elongation at break reached as much as 112%) and avoided secondary crystallization and therefore deterioration of the mechanical properties after at least 4 and 2 months, respectively [56,57]. Other strategies have involved the use of copolymers with controlled monomer content (e.g., PHBV and PHBHHx) [18,30], and of PHB blends with other biodegradable polymers [18,33]. As an example, PHB (naturally isotactic) was blended with synthesized atactic PHB reaching up to 500% elongation at break, making it more flexible and tougher with increasing atactic PHB fraction [58]; however, it is important to note that the other

mechanical properties decreased (Young's modulus and tensile strength were reduced) and that enzymatic degradation was faster [58].

Another aspect to contemplate is that the most common used solvent to process PHB is chloroform, but other more efficient and environmentally friendly solvents can be used, like acetic acid [14]; With the latter solvent, PHB films can be solvent cast and the crystallinity (and mechanical properties) of the films can be modulated by controlling the casting temperature [14].

Efforts have also been made at the industrial level. Biomer has employed strategies to overcome drawbacks in processability and brittleness, boosting properties like creep resistance, easiness to set melt viscosity (due to the linearity of the polymer chains — a forty-fold change in viscosity can be achieved with a difference in temperature of approximately 10 °C), and long term stability to obtain a polymer that can even outperform PP in solidifying speed and impact strength [55]. Some of their strategies include improving thermosensitivity by using solvent instead of water extraction; reducing slow solidification (caused by slow crystallization) by reducing spherulite size using boron nitride as nucleant, setting the crystallization temperature at approximately 90 °C, and decreasing molecular friction of the polymer chains with plasticizers compatible with the crystals [55]. They also reduced brittleness by increasing the amorphous mass, filling the spaces between spherulites with small amounts of compatible polymers [55].

2.3.4. Commercialization and sustainability of PHB

Among the companies pursuing commercial PHA production mentioned in section 2.2, Biomer, Goodfellow, and Tianan are the main suppliers of PHB [25,30,33,35].

Attempts at decreasing PHB production costs and increasing sustainability include varying fermentation methods (anaerobic, continuous/non-sterile) [30], and modelling optimized fermentation strategies [59]. The use of mixed and alternative carbon sources have unfortunately led to low yields [33]. Recent efforts towards these goals have tackled PHB production by methanotrophic bacteria using methane and methanol as feedstock [60,61]. Moreover, in addition to genetically manipulated bacterial strains [19,30], PHB production has been introduced in higher organisms, like yeast and insect cells, but only reaching 0.5% and 0.16% cell dry weight [19,62,63]. Production in plants has rendered better yield (up to 14% and 18% dry weight in leaves of *Arabidopsis thaliana* and tobacco plants, respectively [64,65]). Challenges of PHB production in plants include gene containment, low product yield compared to bacterial systems, and compromised plant health [19,32]. Metabolic engineering approaches have also been attempted, for example by using PHB producing microorganisms as microbial factories for co-production with other value-added products such as rhamnolipids [66], recombinant human tissue plasminogen activator [67], L-tryptophan [68], L-glutamate [69], among others (as reviewed by Kumar and Kim [70]). In addition, the use of clustered regularly interspaced short palindromic repeats interference (CRISPRi) technology in the producer *Halomonas* sp. TD01 has also been explored, allowing to enhance PHB synthesis (diverging more acetyl-CoA from the tricarboxylic acid cycle towards PHB production) and to control copolymer composition of PHBV with the repression of strategic genes [71].

Since PHB is such a promising material, efforts continue to overcome the barriers to widespread usage, taking into account sustainable processes and complete life cycle [1]. In a study performed by Tabone et al., PHAs (including PHB [72]) produced from corn

grain and corn stover were compared to 10 other biodegradable and non-biodegradable polymers. PHB ranked second for green design, and eighth (from corn grain) and fourth (from corn stover) for life cycle assessment [73]. Even though in some instances energy use and CO_2 emissions have been reported to be greater for PHB than for conventional plastics [74], it has been suggested that this could be reverted if all life cycle stages are optimized [75]. An important factor to improve the reliability of life cycle assessments is to better understand the biodegradability of PHB and to devise better waste management processes.

2.4. Degradation of PHB

Polymer degradation can be defined as the process where polymer chains scission occurs, forming oligomers and/or monomers [76]. Chemical degradation via hydrolysis is the most important degradation mechanism in biodegradable polymers [76]; in general, polymer bonds (ester bonds in the case of PHB) can be cleaved by passive hydrolysis or by an active enzymatic reaction [76,77]. For PHB, the enzyme catalyzes the interaction of the polymer chain and water, causing polymer chain breakage and allowing biodegradation to take place [77]. When sufficient external energy exceeding the strength of the bonds is applied, photo, mechanical, and thermal degradation can also occur [76]. However, as mentioned in section 2.3.2, PHB is rather resistant to UV light [5,54,55]. Mechanical degradation plays a role when the polymeric material is exposed to high mechanical stress [76]. For PHB, this situation can occur with applications like sutures [78], or during processing through mixer rotation speed — which influences thermal stability (higher speed, faster and greater thermal degradation) [79].

2.4.1. Thermal degradation

Thermal degradation of PHB has been comprehensively studied with identification and quantification of its products, changes in Mw, and description of the reaction mechanism — non-radical or non-oxidative as it can occur similarly regardless of the atmosphere (nitrogen, air, and vacuum) [18,80-84]. Briefly, random chain scission of the ester bonds occurs just above the polymer melting point (up to 200 °C) with rapid decrease in Mw through a hexa-ring ester intermediate [80,82,84]. Between 250–300 °C, PHB is volatilised resulting in oligomers up to tetramer (dimer and trimer in higher proportion), crotonic acid and isocrotonic acid, in a smaller proportion [81-84]. Between 340–500 °C, acetaldehyde, ketene, propene, carbon dioxide, and β-butyrolactone are formed in small amounts, and traces of carbon monoxide can be detected [82-84].

Other studies have gone more in depth to study factors that can influence thermal degradation. For instance, a study showed that inorganic oxides like CaO, MgO, PbO, PbO_2, $A1_2O_3$, ZnO and calcium hydride catalyze PHB chains splitting, making it easier to form volatile products — by decreasing the temperature of the maximum rate of release of volatile products [85]. Avoiding the presence of such components during extraction can aid in improving thermostability [55]. It has also been shown that fermentation residues have little influence on thermal degradation of PHB, while addition of ammonium surfactants accelerates it significantly [79].

2.4.2. Chemical degradation via hydrolysis

Even though PHB is not prone to rapid abiotic hydrolysis, it can undergo hydrolysis in water through the universal ester acid-base reaction [47]. At 37 °C and neutral pH, this reaction is very slow, as demonstrated by *in vitro* incubation experiments of PHB monofilaments with phosphate buffered saline for up to 180 days, as well as *in vivo* in rats [86]. Only γ-irradiated PHB (in which pre-degradation took place, likely reducing Mw) showed alteration of mechanical properties after 7 days *in vivo* — but not after 14 days *in vitro* [86]. This suggests variable non-specific interactions with enzymes secreted by the immune system (like lysozyme and esterases) take place — since no PHB-utilizing microorganisms are encountered in the body [54,86]. In another study, PHB, PHBV, and P(3HB-co-4HB) films were incubated *in vitro* in 0.0l M phosphate buffer (pH 7,4) at 37 °C for 180 days [87]. The polymers showed a reduction in number-average molecular weight (Mn) but no mass loss after an induction period (likely necessary for water to permeate the polymer matrix) — 80 days incubation for PHB and PHBV, and 20 days for P(3HB-co-4HB) [87]. P(3HB-co-4HB) was additionally tested at 70 °C, where significant mass loss was observed but only after Mn decreased below 13,000 [87]. This suggests hydrolytic degradation occurs first through homogeneous erosion by random hydrolytic chain scission with Mw decrease (accelerated by the presence of 4HB) followed by mass loss [87]. Mergaert et al. also showed that chemical hydrolysis of PHB and PHBV in sterile buffer is slow, since the mass of polymer pieces remained unchanged after incubation at 4–55 °C for 98 days [88]. However, at higher temperatures (40 and 55 °C) there were decreases in Mw (in agreement with the study from Doi et al. [87]), and at 55 °C there was reduction of elongation at break (other mechanical properties did not change) [88].

PHB hydrolysis can also occur through exposure to chemicals, for instance PHB has inferior solvent resistance compared to PP [54]. In addition, acids or alkalis are known to degrade polyesters [5]. For example, exposing PHB granules to a strong acid like 3 N HCl at 104.5 °C resulted in hydrolysis, where the acid attacked components of the crystallized envelope and the amorphous nucleus [5].

2.4.3. Biodegradation

Some microorganisms are able to biodegrade and utilize PHB through the action of intracellular and extracellular PHB depolymerases (PhaZs) — Enzyme Commission Number EC 3.1.1.75 [5,12,13,18]. This process is two to three orders of magnitude faster than regular hydrolytic degradation [89,90] and can take place under a broad range of environmental conditions.

2.4.3.1. Intracellular biodegradation

Intracellular PHB biodegradation consists of active hydrolysis of the endogenous accumulated PHB granules (inclusion bodies) by intracellular PhaZs (i-PhaZs) — also known as mobilization — inside PHB-producing bacteria to retrieve carbon/energy [5,12,13,18]. In comparison to extracellular biodegradation, the mechanism of intracellular degradation is understudied [13,18]. i-PhaZs are only able to degrade amorphous native PHB, not semicrystalline PHB (extracted from the accumulating bacteria) [91]; but an exception is i-PhaZ from *Bacillus megaterium* (PhaZ1), which can degrade native PHB granules (without the aid of an activator or protease to remove the external layer) and semicrystalline PHB into monomeric units [92].

Rhizobium, *Spirillum*, and *Pseudomonas* strains have been reported to synthesize and breakdown intracellular PHB during culture [93]. In the absence of a carbon source, PHB producing bacteria are able to survive due to their production and utilization of PHB [42,94]. *Legionella pneumophila*, for example, was able to survive in tap water for up to 600 days at 24 °C [95], and *Alcaligenes eutrophus* degraded accumulated PHB when carbon sources were absent [42].

The degradation of native PHB granules is thought to involve an activator protein (which can also be replaced by trypsin or alkaline extraction) that renders the amorphous PHB available to the i-PhaZ — which is otherwise inhibited by proteins in the protective layer [96,97]. In some cases, the native PHB granules appear to self-hydrolyze: for example, Uchino et al. have shown that granules from *Ralstonia eutropha* contained PHB biosynthetic proteins, other proteins, and an i-PhaZ [98]. The presence of both synthesis and mobilization enzymes (in this case, the degradation product was the PHB precursor 3HB-CoA) suggested that the processes involved depended on the concentration of intracellular key metabolites [98]. Typically to complete the cycle of PHB metabolism, 3HB monomers are oxidized to acetoacetate by NADPH-dependent 3HB dehydrogenase, then acetoacetyl-CoA is esterified (this can be performed by 3-ketoacid-CoA transferase with CoA supplied by succinyl-CoA, or with acetoacetyl-CoA synthase), and PHB synthesis can start again [43,99].

2.4.3.2. Extracellular biodegradation

Many non-PHB-producing microorganisms can utilize PHB. This is mediated by extracellular PhaZs, enzymes that typically have structures and sequences unrelated to

those of i-PhaZs [31]. Since extracellular PhaZs are the focus of this work, from here onwards PhaZ refers to extracellular PHB depolymerases in this chapter, unless otherwise specified. These enzymes can hydrolyze denatured semicrystalline PHB into water soluble oligomers and monomers, which can then be metabolized [18]. PhaZs expression is usually repressed if another carbon source, such as glucose, is present [13]. A few special cases have been shown: For some strains, since PhaZs excretion continued after growth cessation, the presence of PHB has been proposed to be non-essential for PhaZ induction [100]; and for *P. lemoignei*, PhaZ production is at its peak in batch cultures with succinate [12,100].

- **PhaZs structure**

Structurally, most PhaZs contain multiple domains (Figure 2.2): a signal peptide (SP), a large N-terminal catalytic domain (Cat), a short linker region (link) that binds Cat to the C-terminal portion, and a C-terminal substrate binding domain (SBD) [12,13].

The SP is typically composed of 22 to 58 amino acids and is responsible for secreting the mature PhaZ across the cytoplasm [12,13]. To enable secretion, it is excised by signal peptidases.

Cat has two possible conformations (type 1 and type 2), depending on the arrangement of key residues. As PhaZs are esterases, Cat possesses an active site (catalytic triad) composed of a serine (Ser, S, which is part of a lipase box pentapeptide Gly-Xaa1-Ser-Xaa2-Gly — where Gly is glycine and Xaa represent any amino acid residue and Xaa1 is usually histidine in lipases and leucine in PhaZs), an aspartic acid (Asp, D) and a histidine (His, H), conserved by the α/β-hydrolase fold superfamily [12,13,101]. In addition, a

second conserved His residue — thought to help stabilize the transient state during hydrolysis since the region around it is similar to the oxyanion hole in lipases — which in type 1 Cat is positioned on the N-terminal side of the catalytic triad, and on the C-terminal side in type 2 [12,13,102].

In PhaZs, link has one of three compositions: it can be threonine rich, a fibronectin type III (Fn3), or a cadherin-like sequence [12,13].

The SBD is typically composed of 40 to 60 amino acids and is one of two types, both with conserved His, arginine, and cysteine (Cys) residues, while type 1 SBDs have an additional Cys [12,13].

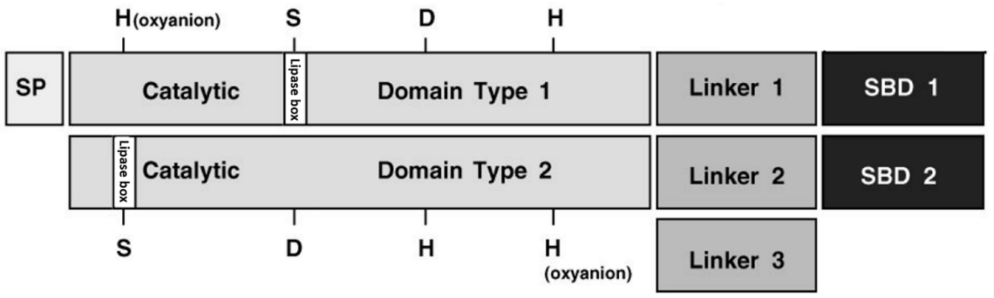

Figure 2.2. Sequence models of domains of PhaZs specific to SCL PHAs.
SP stands for the signal peptide; two possible types of catalytic domain based on the position of a conserved His residue with respect to the catalytic triad; three possible types of linker domains (threonine rich, fibronectin type III, or cadherin-like sequence); and two types of substrate binding domains (SBD). Adapted from [12]. Republished with permission of Annual Reviews, from Microbial Degradation of Polyhydroxyalkanoates, Dieter Jendrossek and René Handrick, 56:1, 403-432, 2002; permission conveyed through Copyright Clearance Center, Inc (Order License Id: 4513710576928).

A few exceptions to these typical conformations have been identified. For instance, two SBDs have been reported for PhaZs from *Alcaligenes faecalis* AE122 (one PHB

specific, the other both PHB and PHV specific, according to protein sequence comparison with other known PhaZs), *Pseudomonas stutzeri*, *Marinobacter* sp. NK-1, and *Bacillus* sp. NRRL B-14911 [103-106]. In the latter bacterium, the two SBDs showed homology to SBDs present in annotated PhaZs of *B. megaterium* QM B1551, *Bacillus pseudofirmus* OF4, and *Bacillus* sp. SG-1 [106]. It also contained two new types of linker domains, without characteristic or amino acid similarities to the three known linker types [106]. The second link domain was similar to domains present in the PhaZs from other *Bacillus* species [106]. This indicates that this PhaZ from *Bacillus* sp. NRRL B-14911 could be a representative of a new class of PhaZs [106]. Another example of non-typical structure was found in PhaZ from *Penicillium funiculosum*, which is composed of a single domain with thirteen exposed hydrophobic residues that could mediate substrate binding [107].

- **PhaZs mechanism**

In general, PhaZs adsorb to the surface of PHB through the SBD (or SBDs), followed by cleavage of the polymer by Cat [108]. Enzymatic hydrolysis of PHB is a surface reaction, where PhaZs can attack the polymer free points [109-111]. PHB crystallinity plays a role in the process as PhaZs hydrolyze the polymer chains in the amorphous regions more easily and then proceed to break the chains in the crystalline phase at a slower rate [89,112]. Studies conducted with single PHB crystals and PhaZs revealed that SBD adsorption occurs homogeneously on the whole crystal surface [113-115]. Degradation however occurs from the crystal edges and ends, rather than at the chain-folding surfaces (where the chain packing regions are disordered), generating narrow cracks (needle-like) and small crystal fragments along the long axis of the crystal [113-115]. An investigation with a mutant PhaZ, not able to hydrolyze PHB, revealed that

fragmentation occurred on the crystal, suggesting there is a disturbance of the polymer chains after adsorption but before hydrolysis occurs [116].

Many other factors influence degradation and its mechanisms. Stereoregularity is an important factor as PhaZs are only able to degrade ester linkages of monomers in the (R) configuration [117,118]. Following typical enzymatic theory, degradation rate also increases to a maximum value with increasing enzyme concentration and then decreases as enzyme inhibition effects take over [109]. Depending if the PhaZ has and endo-, exo-, or combined hydrolase activity, the degradation products can be monomers, dimers, or a mixture of oligomers including monomers (for example, oligomers with one to six 3HB units have been detected for PhaZ7 from *P. lemoignei* after degradation of native PHB [119]) [5,12]. Some bacterial strains also possess extracellular oligomer hydrolases that cleave the oligomers into monomers [120,121].

A detailed catalytic mechanism for the general action of PhaZ has been described by Jendrossek [13], for the specific PhaZ from *P. funiculosum* (PhaZ$_{Pfu}$) by Hisano et al. [107], and for PhaZ from *Ralstonia pickettii* T1 (PhaZ$_{RpiT1}$) (formerly *Alcaligenes faecalis* T1 and currently *Cupriavidus* sp. [122] — see APPENDIX A, section A.4) by Tan et al. [123]. Briefly, after PhaZ binds on the PHB substrate through hydrophobic interactions and the formation of hydrogen bonds, the Ser residue of the catalytic triad acts as a nucleophile on the carbonyl carbon atom of the PHB chain. This is facilitated by the His residue — its imidazole ring enhances the reactivity of the oxygen atom of the Ser residue and the acidity of the hydroxyl-function — while the carboxyl group of Asp stabilizes the positive charge of the imidazole ring. This is followed by the attack of a molecule of water on the ester bond (Figure 2.3) [13,107,123].

In the PhaZ$_{Pfu}$ model, a carbonyl group of a monomer unit next to the ester linkage being hydrolyzed forms a hydrogen bond with a tryptophan residue to permit the attack by Ser, the next monomer unit forms hydrogen bonds with two asparagine (Asn) residues through a water molecule, and the methyl groups of those monomeric units are bound to hydrophobic pockets of the enzyme [107]**.** An oxyanion hole may stabilize a transient tetrahedral coordination of the carbonyl carbon atom (with the formation of a covalent acyl-enzyme intermediate) [107]. This phenomenon would be analogous to the role of an oxyanion amino acid in lipases [12,13,102,107]. In the mechanism of PhaZ$_{Pfu}$ presented by Hisano et al., the side chain of His does not point to the active site, so this role would be played by the amide groups of Ser or Cys [107]. This would allow the formation of a hydrogen bridge to the negative oxygen (oxyanion) of Ser [12,13,102,107]. Tan et al. proposed that in addition, another Asn in Cat might participate in substrate recognition, since mutated versions of PhaZ$_{RpiT1}$ for this residue decreased PHB degradation efficiency but improved hydrolysis of *p*-nitrophenyl esters [123].

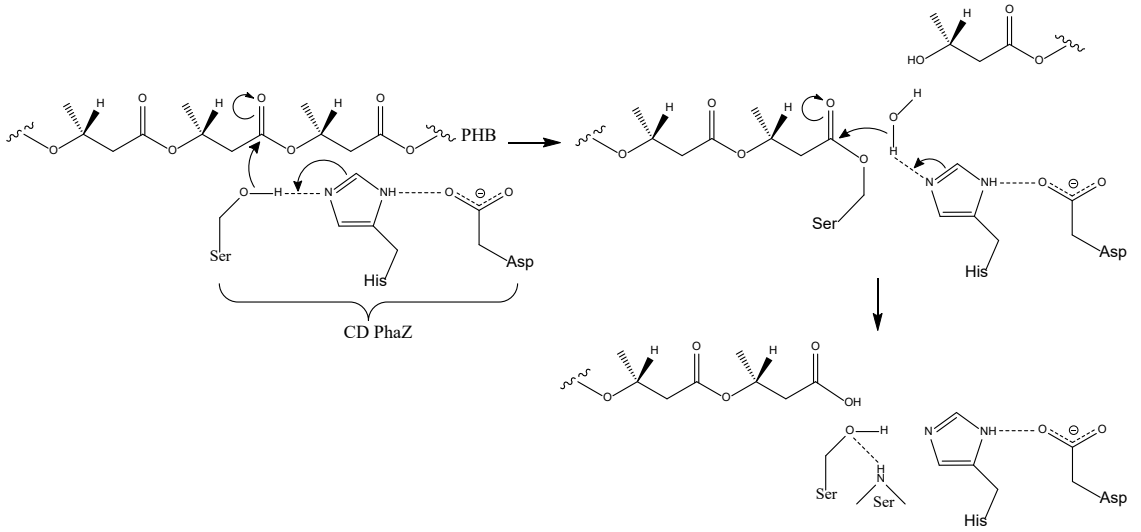

Figure 2.3. Summarized mechanism of PHB degradation by PhaZs.
Ser, Asp and His constitute the active center of the PhaZ catalytic domain (CD in the figure). Based on [13,107,123].

2.4.3.3. Biodegradation under environmental and laboratory conditions

Since it can take months to years for PHB to degrade depending on the environmental conditions (including pH, temperature, humidity, presence of ions, and nutrients) and microflora [88,103,124,125], it is important to consider the impact of these factors on the efficiency and capability of PhaZs.

The biodegradation of PHB (and of other PHAs) has been reported in different environments and under various laboratory conditions with soil, sewage sludge, compost, fresh water, marine-related ecosystems, among others (as reviewed by [5,18]). One of these studies investigated PHB and PHBV degradation in different soils at 15, 28, and 40 °C for up to 200 days in a laboratory [88]. Controls were incubated in sterile buffer at temperatures between 4 and 55 °C for 98 days [88]. A variety of microorganisms — 295 strains were

identified, including several Gram-negative bacteria (mostly *Acidovorax facilis* and *Variovorax paradoxus*) and Gram-positive *Bacillus* strains, *streptomycetes*, and molds (*Aspergillus fumigatus* and species of the genus *Penicillium*) — were able to degrade the samples, and their action was enhanced at higher temperatures [88]. At 40 °C, Mw decrease occurred to the same extent in samples and in sterile buffer controls, but mass loss only took place in soils (0.03–0.64% mass loss/day, dependent on type of soil, temperature), and PHBV generally degraded faster [88]. In addition, a different study focused on degradation of ultra-high-Mw PHB films in river water at 25 °C under modified standardized test conditions [126]. In this case, degradation was noticeable after 4 days and complete degradation took place over three weeks [126].

The biodegradation of PHAs, including PHB, in marine environments is of special interest given the current challenges with plastic accumulation in the oceans. Such investigations have been carried out by Doi et al. [127], who found that sample erosion occurred regardless of copolymer composition of the polyesters studied but was dependent on seawater temperature. The authors also isolated two *Streptomyces* strains able to use PHB [127]. In another study, the degradation of P(3HB-co-14%3HV) fibers was studied in controlled reactor conditions with different natural waters, including seawater [128]. Several *Pseudomonas* strains, among others, were able to grow well using PHB or P(3HB-co-14%3HV) as sole carbon source [128]. Other studies included investigating PHB and PHBV films degradation in tropical seawaters of the South China Sea [129-131]. The bacteria responsible for the mass loss of both PHAs were of the *Enterobacter*, *Bacillus* and *Gracilibacillus* genera [129-131]. PHB degradation has even been studied under simulated deep sea pressure conditions with deep sea yeast (*Rhodosporidium sphaerocarpum*) and

fungus (*Aspergillus ustus*) isolates, two marine surface yeast isolates (*Candida guilliermondii*, *Debaryomyces hansenii*), and one terrestrial fungus (*Aspergillus ustus*) [132]. While all strains could degrade PHB at atmospheric pressure, increasing pressure limited metabolic, growth, and degradation capacities for all strains studied; with no activity detected above 30 MPa [132]. A similar study showed yeast and fungi isolated from marine habitats could degrade PHB and a commercial version of PHBV, BIOPOL™ [133,134]. Only 4.5% of the tested strains could degrade PHBV, and 6.7% PHB — *Asteromyces cruciatus*, *Candida guilliermondii*, *Debaryomyces hansenii*, *Nia vibrissae*, and *Rhodosporidium sphaerocarpum* were identified among the degraders [133,134].

2.4.3.4. Extracellular PhaZs survey

Most studied PHB-degrading microorganisms have been isolated from environmental samples incubated on agar plates containing PHB as the sole carbon source [13]. The first study on extracellular PHB degradation was published in 1963 by Chowdhury ([135] as reviewed by [13]). Since then, many microorganisms and PhaZs have been studied and characterized. The PHA Depolymerase Engineering Database (PHA-DED) was developed by Knoll and coworkers in an effort to create a complete repertory of PhaZ sequences [136]. This database is currently available as a webserver (http://www.ded.uni-stuttgart.de/) [136]. At the time of its release in February 2009, 587 PHA depolymerase sequences had been compiled, but only 28 of these had been experimentally demonstrated to have PhaZ activity [136]. The PHA-DED serves as a tool to predict PhaZ sequences from genomic data; from the 28 validated PhaZs, 12 sequences belonged to SCL PhaZs with Cat type 1, and 7 with Cat type 2 [136]. In addition, an

excellent overview of PHA degrading microorganisms and biochemical characteristics of purified PhaZs was compiled by Jendrossek [13]. Both these efforts can be reviewed for information on PhaZs from, among others, *Comamonas testosteroni* 31A [137,138], *Cupriavidus* sp. [139-141], *Pseudomonas stutzeri* [105,142], *Ralstonia* sp. [139,143,144], and the complete system of 7 PhaZs from *Paucimonas lemoignei* [145-150] — which includes the exceptional PhaZ7, an extracellular PhaZ able to degrade amorphous PHB [146,148]. In 2017, a review on enzymatic degradation of biodegradable plastics was published by Roohi et al. [151]; it offers an overview of purified depolymerase enzymes, including extracellular PhaZs able to degrade PHB numbered 1–7 (8 is a periplasm-associated PhaZ from *Rhodospirillum rubrum* specific for amorphous PHB [152]), 9–15 (with a reference mistake for 10, PhaZ from *Agrobacterium* sp. DSGZ [153]), and 27–29 [151]. Some of the PhaZs are from *Pseudomonas mendocina* DSWY0601 [154], the fungal isolate *Emericellopsis minima* W2 [155], *Acidovorax* sp. DP5 [156], and the previously mentioned *Bacillus* sp. NRRL B-14911 with two link domains and two SBDs [106]. Other representatives of extracellular PhaZs specific to semicrystalline PHB, additional to the ones mentioned in the referred resources [13,136,151], are compiled in Table 2.2 below (along with characteristics like size of the mature peptide determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or liquid chromatography-tandem mass spectrometry (LC-MS/MS)).

Table 2.2 Overview of purified PhaZs, microorganisms and some characteristics. Complementary to reviews in [13,136,151].

Strain, PhaZ name (gene)	Mature protein Mw [kDa]	Characteristic and remarks	PhaZ GenBank, reference	Journal, publisher, year
Aspergillus fumigatus 76T-3	57 (SDS-PAGE, LC-MS/MS)	Optimum conditions: pH 6.4 and 55 °C; purified version can also degrade polyethylene succinate and polybutylene succinate, but the recombinant PhaZ expressed in *E. coli* cannot	[157]	Polym Degrad Stab, Elsevier, 2018
Aspergillus sp. NA-25	48, 75, 80, and 85 (SDS-PAGE)	Produced four PhaZs; stable at pH 6.0–9.0 and 37–60 °C	[158]	Int J Biosci, INNSPUB, 2015
Bacillus megaterium N-18-25-9, $PhaZ_{Bm}$ ($phaZ_{Bm}$)	62.3 (SDS-PAGE)	Optimum conditions: pH 9.0 and 65 °C; C-terminal region only similar to PhaZ from *Bacillus* sp. NRRL B-14911	AB258388, [159]	FEMS, Oxford University Press, 2006
Burkholderia cepacia DP1	53.3 (SDS-PAGE)	Optimum conditions: pH 6.0 and 40 °C; PhaZ production enhanced through response surface methodology	MH70889, [160,161]	3 Biotech, Springer 2017 Protein Expr Purif, Elsevier 2019
Caldimonas manganoxidans, $PhaZ_{Cma}$ ($phaZ_{Cma}$)	46 (SDS-PAGE)	Catalytic activity at 70 °C	AB038647.1, [162]	J Biosci Bioeng, Elsevier, 2000
Diaphorobacter sp. PCA039, PhaZd (*phaZd*)	50 (SDS-PAGE)	Optimum conditions: pH 8.0 and 40 °C; also degrades PHBV	[163]	World J Microbiol Biotechnol, Springer, 2010
Fusarium solani Thom, $PHAZ_{Fus}$	85 (SDS-PAGE)	Optimum conditions: pH 7.0 and 55 °C; partial purification was performed	[164]	J Chem, Hindawi, 2012
Paecilomyces sp. DS1407	55 (SDS-PAGE)	This is a P(3HB-co-4HB) depolymerase, but degrades PHB and PHBV as well with 42 and 70% less specific activity compared to P(3HB-co-4HB)	[165]	Polym Degrad Stab, Elsevier, 2019
Penicillium expansum, $PhaZ_{Pen}$	20 (SDS-PAGE)	Optimum conditions: pH 4.0–6.0 and 45–50 °C; partial purification was performed	[166]	3 Biotech, Springer, 2015
Shewanella sp. JKCM-AJ-6,1α, $wPhaZ_{She}$ [wild type], $rbPhaZ_{She}$ [recombinant], ($phaZ_{She}$)	47 and 70 (SDS-PAGE)	$wPhaZ_{She}$ unstable above 15 °C and relatively high enzymatic with 0.5 M NaCl; presence of two SBDs, that were missing in the wild type PhaZ resulting in less binding affinity but avoiding self-inhibition, contrary to recombinant PhaZ	[167]	Polym Degrad Stab, Elsevier, 2016
Streptomyces ascomycinicus, $PhaZ_{Sa}$ (*fkbU*)	48.4 (MALDI-TOF)	Optimum conditions: pH 6.0 and 45 °C; first PhaZ from a Gram-positive bacterium with acidic pH optimum	AAF86381.1, [168]	PLOS ONE, PLOS, 2013
Streptomyces lydicus MM10	65 (SDS-PAGE)	Optimum conditions: pH 8.0 and 45 °C	[169]	Int J Agric Biol, FSP, 2015
Streptoverticillium kashmirense AF1	37 and 45 (SDS-PAGE)	Produced two PhaZs; optimum PhaZs production pH 8 and 7, at 45 °C, 1% substrate and presence of lactose as additional carbon source	[170]	Acta Biologica Hungarica, AK Journals, 2008
Talaromyces verruculosus	45 (SDS-PAGE)	During culture, enzyme production was highest at 30 °C at early growth stage	[171]	Research J Pharm and Tech, A and V Publication, 2014

2.4.3.5. Recombinant production of PhaZs

PhaZs have been purified from wild-type strains usually through multiple steps of affinity purification, including hydrophobic interaction chromatography [12]. Such purification often requires large amounts of culture grown under conditions favouring the production of PhaZ, which in some instances can be challenging. Another alternative is to make use of recombinant protein technologies, to overexpress the *phaZ* gene.

The production and characterization of recombinant PhaZs (rPhaZs) require multiple steps laid out in Figure 2.4. Once a microorganism is identified as a PHB degrader (usually from screening of environmental samples with PHB plates [13]), its *phaZ* gene must be identified and recovered. Strategies have included design and production of DNA probes from known *phaZ* gene sequences — through polymerase chain reaction (PCR) — or by using degenerate primers, if the N-terminal amino acid sequence of PhaZ purified from the wild-type microorganism was previously obtained [104,105]. The probes are then hybridized to genomic DNA (gDNA) digested with specific restriction enzymes, and positive DNA fragments are inserted into vectors and cloned for screening [104,105]. With advances in next-generation sequencing, whole genome sequencing of known or potential degraders allows to analyze and annotate genes with predicted PhaZ functionality. The gene sequences thus identified, can be used to design primers to amplify and recover the genes through PCR. This strategy was employed to produce rPhaZ from *Pseudomonas mendocina* DSWY0601 from genomic data [154]. Once the *phaZ* gene has been amplified, restriction digestion followed by ligation into a vector (plasmid) is performed. Cloning is the next step, where a suitable cloning host allows to obtain copies of successful constructs

that carry the *phaZ* gene; successful clones are usually selected through antibiotic resistance (conferred by another gene built in the vector), and are confirmed by plasmid extraction, restriction digestion and DNA sequencing. In some cases, the cloning host can directly express rPhaZ, and screening can be done by plating the host — when the rPhaZ can be secreted, possible if the SP from the PhaZ to be produced functions correctly in the host, like in the case of PhaZ from *P. stutzeri* in *E. coli* DH5α [105] — or its lysate on PHB plates, and identifying clear zones indicative of polymer degradation. The approach of plating transformants was taken for rPhaZ from *Bacillus megaterium* N-18-25-9 screened using *E. coli* XL10-Gold [159]. However, most of the time, PhaZ is not readily expressed or expression is limited, and the *phaZ* construct must be moved to a host specifically optimized for expression, as described below.

Once the successful *phaZ* construct is obtained, it can be transformed into a host optimized for protein expression (a different expression vector can also be used, see Table 2.3) — usually modified strains of *E. coli*, but yeast [107] and bacterial strains like *Bacillus subtilis* WB800 [148,172] and *Rhodococcus* sp. T104 [173] have also been employed. There is a high number of options when selecting an expression system, and heterologous protein characteristics (e.g. rare *E. coli* codons and the presence of disulfide bonds in the protein tertiary structure) should be considered. Multiple conditions (vector, host, culture conditions) are usually tested to obtain a specific protein [174]. A successful expression host colony carrying the *phaZ* construct is cultured, and the addition of a proper inducing agent activates the inducible promoter within the vector, causing rPhaZ expression. A common compound used for induction of rPhaZ expression is isopropyl-β-d-thiogalactopyranoside (IPTG) — see Table 2.3. After the induction incubation period (at

an appropriate temperature), the culture is harvested through centrifugation and proteins can be extracted (pellets can also be stored frozen for extraction at a later time). Purification can be performed through hydrophobic chromatography, or by incorporating affinity tags to the *phaZ* gene, such as His-tag which has affinity towards metal ions like nickel. His-tags have been used to purify rPhaZs in previous studies [154,159,172]. Purified rPhaZ can be analyzed through SDS-PAGE to verify protein size and estimate purity [175], protein assays like Bradford to quantify concentration [176], and tested for PHB degradation activity.

Some of the described methods are covered in more detail in Chapter 4.

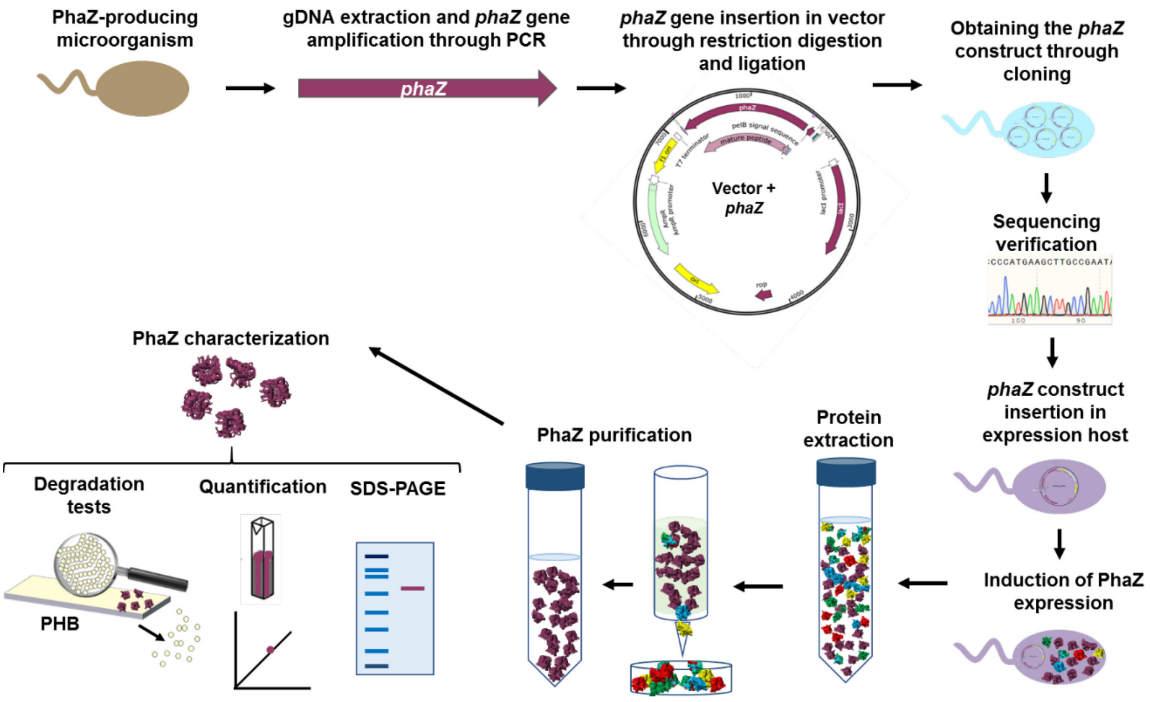

Figure 2.4. Generalized pipeline for production and characterization of rPhaZs.

Another advantage of producing rPhaZs is the possibility of making modifications to the gene structure in order to characterize and/or improve its function, or to obtain greater

quantities for determination of crystal structures [107,172]. Under some circumstances, the rPhaZ may not exhibit the same characteristics as the PhaZ expressed in the wild-type strain. For example, rPhaZ from *A. fumigatus* (Table 2.2) can only degrade PHB, while it was shown to degrade PHB, PES, and PBS when expressed in the wild-type strain [157]. The authors also highlighted a difference in the Mw of the enzyme [157]. They speculated that this was due to a glycosylation pattern in the wild-type fungus strain which was not conserved when expression took place in the expression strain [157].

Examples of rPhaZ production and some of the strategies used are provided in Table 2.3.

Table 2.3 rPhaZs strategies examples.

Strain of origin	Gene	Vector(s) (C:cloning, E:expression)	Host(s) (C:cloning, E:expression)	Induction conditions (I) (when applicable) and purification method (P)	Notes	PhaZ GenBank, reference
Alcaligenes faecalis AE122	$phaZ_{AfaAE122}$	pUC19 (C), pKK223-3 (E)	*E. coli* JM109 (C, E)	Multiple steps, including Diethylaminoethyl cellulose (DEAE)-cellulose DE52, butyl-Sepharose CL-4B, and DEAE-Toyopearl pack 650 columns		[103,177]
Bacillus megaterium N-18-25-9	$phaZ_{Bm}$	pUC19 (C), pQE80L (C, E)	*E. coli* XL10-Gold (C), *E. coli* Origami B (DE3) (E)	I: 0.5 mM IPTG, 16 h at 16 °C. P: His-tag	Production of another PhaZ variant was also done	AB258388, [159]
Caldimonas manganoxidans	$phaZ_{Cma}$	pCR2.1-TOP0 (C), pKK223-3 (E)	*E. coli* TOP10 (C), *E. coli* JM109 (E)	Not performed	For this study the strain was denominated HS, later on denominated *C. manganoxidans* [178]	AB038647.1, [162]
Marinobacter sp. NK-1	$phaZ_{Msp}$	pUC18 (C), pGEX-4T-1 (E)	*E. coli* DH5, *E. coli* XL1-Blue MRF'	I: 0.05 mM IPTG, 4 h at 25 °C. P: GST-tag	Production of PhaZ variants was also done	AB079799.1, [104,179]
Pseudomonas lemoignei	*phaZ1-phaZ5*$_{Ple}$	(pUC9, pUC9-1, pUC9-2)[1,2], pBluescriptSK−(C)[2], pVK100[2]	*E. coli* JM83 (C, E)[1,2,3]; *E. coli* XL1 blue (C, E)[2], *E. coli* S17-1(C, E)[2], *A. eutrophus* H16 and JMP222 (E of $PhaZ1_{Ple}$)[2]	I: 0.2 mM IPTG (*E. coli* JM83, *E. coli* XL1 blue)[2] P ($PhaZ1_{Ple}$[1,2], $PhaZ2_{Ple}$[2], $PhaZ4_{Ple}$[2], $PhaZ5_{Ple}$[2]): Multiple steps, including DEAE-Sephacel and carboxymethyl Sepharose CL6B columns; additional step for $PhaZ4_{Ple}$ with Superdex 200 HR 10/30 column		1: [147] 2: [145] 3: [180]
Pseudomonas lemoignei	$phaZ7_{Ple}$	pWB980 (C, E)[1, 2], pBluescriptSK−(C)[2]	*E. coli* DH5α (E, low amount)[1], *Bacillus subtilis* WB800 (E)[1,2], *E. coli* Xl1-Blue (C)[2]	P: Not performed[1], His-tag[2]	Production of mutants was also done[1, 2], and the crystallized structures of wild-type and mutants were obtained[2]	AY026355.1, [146] 1: [148] 2: [172]
Pseudomonas mendocina DSWY0601	$phaZ_{pm}$	pET-22b (C, E)	*E. coli* DH5α (C), *E. coli* Rosetta (E)	I: 1 mM IPTG, 5 h at 37 °C. P: His-tag		[154]
Pseudomonas stutzeri	$phaZ_{Pst}$	pUC118 (C), pGEX-4T-1 (E)	*E. coli* DH5α (C, E)	I: 0.05 mM IPTG, 4 h at 25 °C. P: Glutathione S-transferase (GST)-tag	Production of PhaZ variants was also done	AB012225.1, [105,179]

2.4.3.6. Assessment of PhaZ activity

PhaZ activity has been studied by several research groups using diverse approaches and methods, making comparisons between studies challenging. Since factors such as PHB crystallinity and Mw — in addition to the typical parameters investigated during enzymatic activity studies (temperature, pH, ionic strength, etc.) — play important roles in the degradation rate, comparisons between studies are especially difficult.

- **Assessing activity using PHB granules**

Many studies monitor the decrease in optical density (OD) of suspensions of PHB granules upon PhaZ exposure as a measure of activity [96,100,104,137,141,145,146,150,181]. This method has some limitations as decreases in OD result from both enzymatic activity and granule precipitation, generally leading to overestimations of activity. Another method, involving quantification of NAD- (diphosphopyridine nucleotide (DPN) in the paper) dependent 3HB was developed [182], but it is time consuming and when oligomers are the product of hydrolysis, they have to be cleaved [31]. Also, granules suspensions are limited by very diluted amounts of available substrate, with the possibility of underestimating high activities due to limited surface area [31]. This restriction seems to be circumvented by employing a pH stat method, through the detection of released acid from the hydrolysis of the ester bonds in a non-buffered solution — pH is kept constant by online pH sensing and pumping a base with its consumption being proportional to PhaZ activity [31]. This method is described as very sensitive, and high amounts of PHB granules can be used, but corrections need to be done for carbon dioxide solubilization or sealed systems need to be used [31].

- **Assessing activity using PHB films**

Another approach involves the use of PHB films, for which the area to volume ratio and possibly the crystallinity would be different. Granules often display a range of parameters that affect the available surface [109], and that are difficult to measure — particle size distribution, roughness, porosity, crystallinity, and others. Therefore, enzymatic activity measurements reported between studies are often not comparable. To this day, most of the studies carried with PHB suspensions or films also use different testing conditions (such as PhaZ concentration, medium, temperature, pH, PHB source and characteristics, and rate determination) and evaluate a single PhaZ; few comparative studies are available [109,144,180]. A more accurate unit of measurement, for example, would be (PHB mass degraded)/(protein concentration·surface area·time), a measure more easily and reliably obtained with films.

PHB film degradation has most commonly been assessed using gravimetric methods [90,183], This method provides useful qualitative or semi-quantitative information but it is difficult to quantify rates as days or even weeks are often required to observe significant mass losses [31,109].

Spectrophotometric assays have also been proposed. Mukai et al. (1993) measured the kinetics of PhaZs by monitoring the formation of 3HB in solution at 210 nm [109]; this is one of the few comparative studies performed, with three different PhaZs assessed [109]. Kasuya et al. (1995) employed the same method but included the extinction coefficients of both 3HB monomer and dimer, and confirmed the results at the end of the reaction by high-performance liquid chromatography (HPLC) and PHB film mass loss [111]. This method was further modified by Scandola et al. (1998) by stirring the solution and lifting the PHB

film to maximize polymer exposure [184]. In this case, the concentration of 3HB was also estimated from the extinction coefficient of 3HB [184]. Critics of the spectrophotometric method using PHB film include low surface areas and potential detection of impurities at 210 nm that can decrease sensitivity [31]. These can be avoided by using enzyme concentrations that prevent issues of substrate availability (also a concern with PHB granules [31]), and by performing negative controls under the same conditions without PhaZ.

A method involving pH measurements of solutions during degradation of PHB film was also proposed [185], but pH was not kept constant, affecting PhaZ activity [109]. Perhaps an adaptation of the pH stat method described for granules [31] could be a more suitable solution.

Several related research groups in Japan have taken advantage of the sensitive quartz crystal microbalance (QCM) technique to measure small changes in mass — resolution in the range of ng/cm^2 [186] — to study the adsorption of PhaZs on PHB films and the ensuing degradation [186-188]. Another method, without the need of expensive equipment or extensive sample preparation, includes degradation measurements by diffraction changes in patterned PHB films [189]. The grating changes with surface degradation, and a laser and a photodetector combination can be used to measure the extent of degradation [189]. Of course, microscopy methods like scanning electron microscopy (SEM) [50,126,128,157,165,183,190-193] and atomic force microscopy (AFM) [99,188,189,194,195] can also be employed to assess PHB film degradation, but these are more qualitative.

- **Characterization of degradation products**

The characterization of PHB degradation products resulting from PhaZ activity has been explored, among some methods, with proton nuclear magnetic resonance (H-NMR) [144] and HPLC [117,119]. The method proposed by Gebauer and Jendrossek, in which 3HB oligomers are derivatized to allow sensitive UV detection through HPLC, is relatively easy to perform [31,119]. Gas chromatography (GC) does not seem to be appropriate since oligomers are converted to monomers in the derivatization procedure [31].

2.4.3.7. Examples of PHB-PhaZs technologies

PhaZs enable the development of innovative applications. One case is the potential recycling of PHB. Lee and coworkers proposed an improved whole-cell bioplastics degradation system through co-expression of a PhaZ (that can degrade PHB and at least three other substrates) with a protein that aids secretion [178]. The production of 3HB for the chemical and pharmaceutical industries can also be optimized through the development of improved biocatalysts (avoiding use of solvents and contaminant catalysts, or endotoxins from microorganisms) [196]. For this application, PhaZ was immobilized on magnetite-based nanoparticles for PHB degradation under acidic conditions [196]. A nutraceutical application for PHB and 3HB as components of animal (from cattle to even humans) feed has also been proposed as these compounds were shown to help modulate gut flora and supress or inhibit pathogenic bacteria in the gastrointestinal tract [197]. This technology can be used alone or in combination with suitable PhaZs or PhaZ-producing bacteria that could help release the monomer in the gastrointestinal tract [197]. Biosensors

are another area with immense potential. PhaZ-based dye-loaded PHB time-temperature indicators [198] and microbial detection based platforms have been proposed [199].

2.5. References

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3. Polyhydroxybutyrate Biodegradation Using Bacterial Strains with Demonstrated and Predicted PHB Depolymerase Activity*

3.1. Abstract

The biodegradation of polyhydroxybutyrate (PHB) has been broadly investigated, but studies typically focus on a single strain or enzyme and little attention has been paid to comparing the interaction of different PHB depolymerase (PhaZ)-producing strains with this biopolymer. In this work, we selected nine bacterial strains — five with demonstrated and four with predicted PhaZ activity — to compare their effectiveness at degrading PHB film provided as sole carbon source. Each of the strains with demonstrated activity were able to use the PHB film (maximum mass losses ranging from 12% after 2 days for *Paucimonas lemoignei* to 90% after 4 days for *Cupriavidus* sp.), and to a lower extent *Marinobacter algicola* DG893 (with a predicted PhaZ) achieved PHB film mass loss of 11% after two weeks of exposure. Among the strains with proven PhaZ activity, *Ralstonia* sp. showed the highest specific activity since less biomass was required to degrade the polymer in comparison to the other strains. In the case of *Ralstonia* sp., PHB continued to be degraded at pH values as low as pH 3.3 – 3.7. In addition, analysis of the extracellular fractions of the strains with demonstrated activity showed that *Comamonas testosteroni*,

* A version of this chapter has been published. Adapted by permission from Springer Nature: Springer Nature. Applied Microbiology and Biotechnology. Polyhydroxybutyrate (PHB) biodegradation using bacterial strains with demonstrated and predicted PHB depolymerase activity. Diana Isabel Martínez-Tobón, Maryam Gul, Anastasia Leila Elias, Dominic Sauvageau. Copyright (2018). License Number: 4516730479444.

Cupriavidus sp., and *Ralstonia* sp. readily degraded both PHB film and PHB particles in agar suspensions. This study highlights that whole cell cultures and enzymatic (extracellular) fractions display different levels of activity, an important factor in the development of PHB-based applications and in understanding the fate of PHB and other PHAs released in the environment. Furthermore, predictions of PhaZ functionality from genome sequencing analyses remain to be validated by experimental results; PHB degrading ability could not be proven for three of four investigated species predicted by the polyhydroxyalkanoates (PHA) depolymerase engineering database.

Keywords: Polyhydroxybutyrate (PHB), PHB depolymerase (PhaZ), comparative polymer biodegradation, microbial and enzymatic activity, *Comamonas testosteroni* 31A, *Cupriavidus* sp., *Ralstonia* sp., *Marinobacter algicola* DG893.

3.2. Introduction

The use of biodegradable polymers continues to gain attention for a broad range of applications, from food packaging products with lower environmental impacts [1] to biomaterials used as temporary implants [2]. Regardless of their intended use, it is crucial to understand, enhance and ultimately control the degradation of biopolymers. Polyhydroxyalkanoates (PHAs) are a promising class of natural polymers with properties similar to synthetic polymers such as polypropylene or polyethylene [3]. Since they are produced from renewable resources and can be completely bioassimilated or biodegraded into products innocuous to the environment [3,4], PHAs are a promising sustainable solution to the growing problem of the environmental accumulation of plastics. This accumulation has reached a point where even remote uninhabited islands in the South

Pacific have become the destination of growing amounts of plastic waste in the ocean [5]. Polyhydroxybutyrate (PHB), in particular, is the most commonly used PHA [6] and is, generally, industrially produced from sugars [4].

The biodegradation of PHAs — among them PHB — has been studied with diverse intracellular and extracellular hydrolase enzymes that are part of a class of enzymes called PHA depolymerases (PhaZs – Enzyme Commission number EC 3.1.1.75 [7]), which are produced by different microorganisms [6]. Extracellular PhaZs hydrolyze semi-crystalline PHA (with one known exception that hydrolyzes amorphous PHB [8]), producing monomers and oligomers which can be processed by microorganisms as carbon sources [6,9]. Under aerobic conditions, these metabolites are then converted to energy, carbon dioxide and water, or, under anaerobic conditions, to energy, methane and water [6,9]. The rate at which PhaZs degrade PHB depends on the enzymes sequence, conformation and concentration, as well as the environmental conditions, and the polymer itself. It can take a relatively long time to degrade a small piece of PHB if conditions such as temperature and state of the ecosystem are not adequate [3]. For example, it is possible for PHB to degrade within months (in anaerobic sewage) to years (in seawater), as reviewed by Madison and Huisman [10] from several investigations [11-14]. On the other hand, a study showed that similar levels of degradation could be obtained for both writing paper and PHA thin films (PHB and polyhydroxybutyrate-co-hydroxyvalerate were studied) placed in tap water containing aerobic microflora, after 40 days of exposure at room temperature ([15] reviewed in [3]).

As of February 2009, 587 PhaZs sequences had been compiled in the PHA Depolymerase Engineering Database (PHA-DED), but only approximately 30 of these had

been experimentally demonstrated to have PhaZ activity [16]. In the case of the strains investigated in this work, focused on PHB degradation, some of the PhaZs' data was published as far back as 1982 [17], which can lead to confusion when strains are renamed or reclassified. Additional work is required both to demonstrate the activity of predicted strains experimentally, and to compare the effectiveness of different strains under similar conditions. Such comparisons could inform both better predictions and applications, such as the development of specific microbial communities with high PHB degradation capacity.

By comparing the degradation of PHB films by different bacterial strains — some with demonstrated and others with predicted extracellular PhaZ activity based on genomic analysis [16] — and their extracellular fractions, this study provides valuable information related to the nature and activity of PhaZ enzymes, while obtaining experimental results, that points towards confirming the theoretical extracellular PhaZ activity of one bacterial strain (since extracellular PhaZs are the focus of this work, from here onwards PhaZ refers to extracellular PHB depolymerases in this chapter, unless otherwise specified). The strains with predicted PhaZ activity selected were all from marine environments, such as seawater or marine basins. This is of interest since PHB is expected to be present in significant quantities in these ecosystems. Comparisons were based on gravimetric measurements of PHB films in contact with bacterial cultures or extracellular fractions containing excreted PhaZs, and on the degradation of solid media containing suspended PHB powder. In addition, bioinformatics analysis comparing the predicted and demonstrated PhaZs selected for this study was performed to provide insight on how protein sequences can impact degradation. These results can serve as a basis for the determination of the potential

fate and rate of degradation of PHB in the environment, and the utilization of bacteria and PhaZs for treatment of PHB.

3.3. Materials and methods

3.3.1. PHB film fabrication

PHB pellets (BRS Bulk Bio-pellets, Bulk Reef Supply, Golden Valley, USA) and acetic acid (Fisher Scientific) were used to produce PHB films by solvent casting as described by Anbukarasu [18]. The selected solvent casting temperature was 140 °C, and the resulting film was washed in Milli-Q water, left to air dry and age for at least one week, and autoclaved before being used. PHB films produced using this technique have a crystallinity of approximately 70%, good thermal stability, and mechanical properties comparable to chloroform-cast films, but with the advantage of using a more efficient and environmentally friendly solvent [18].

Weight-average molecular weight (Mw) and polydispersity index (Mw/Mn) of the untreated PHB pellets and film (reprecipitated PHB pellets, dissolved in boiling acetic acid at 118 °C for more than 8 hours) were determined using a gel permeation chromatography (GPC) system equipped with a refractive index (RI) detector (Agilent Technologies, 1100 series), using a PL MIXED-B-LS separation column, 300x7,5mm und 10µm PSgel (Agilent Technologies), and chloroform (1.0 ml/min flow rate) as the GPC eluent.

3.3.2. Selection of strains and PhaZs

The strains used in this study are listed in Table 3.1; the microorganisms' suppliers were the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ) and the Japan Collection of Microorganisms (JCM). The strains were selected for their demonstrated or predicted PHB depolymerase activity. The strains with previously demonstrated PhaZ activity were selected for their high activity as reported in the literature and their range of effective conditions (pH and temperature ranges over which PhaZ activity was demonstrated). The strains with predicted PhaZ activity were identified from the PHA-DED — selected from extracellular denatured short chain length PHAs (e-dPHAscl) depolymerases catalytic domain type 1 (*Loktanella vestfoldensis, Marinobacter algicola* DG893, and *Oceanibulbus indolifex* Hel45), and catalytic domain type 2 (*Alteromonas macleodii*) families — [16] and the World Register for Marine Species [19]. Table 3.2 highlights some of the major characteristics and properties of the PhaZs with demonstrated activity.

Table 3.1. Bacterial strains and media.

Bacterial strain	Recommended growth conditions	Isolated from	Source	Collection number	References
Comamonas testosteroni 31A	Tryptic soy broth, 30 °C	Soil from a greenhouse	DSMZ	6781	[20,21]
Cupriavidus sp. (Catalogued as *Alcaligenes faecalis* until December 2015)	Nutrient broth, 30 °C	Activated sludge obtained from the Toba sewage-treatment plant, Kyoto, Japan	JCM	10169	[17,22]
Paucimonas lemoignei	R2A, 30 °C	Soil, poly-ß-hydroxybutyrate enriched	DSMZ	7445	[23]
Pseudomonas stutzeri	Nutrient broth, 30 °C	Seawater, Jogashima, Kanagawa Pref., Japan	JCM	10168	[24,25]
Ralstonia sp. (Catalogued as *Ralstonia pickettii* until January 2016)	Nutrient broth, 30 °C	Atmosphere in the laboratory, Japan	JCM	10171	[26]
Alteromonas macleodii	Marine broth, 25 °C	Seawater, HI, USA Equivalent to ATCC 27126	JCM	20772	[16] GenBank: AFS36858.1 UniProt accession number (most similar): A0A126PY91
Loktanella vestfoldensis	Marine broth, 25 °C	Microbial mat from Ace Lake in the Vestfold Hills, Antarctica	JCM	21637	[16] GenBank: EAQ05513.1 UniProt accession number: A3V8T2
Marinobacter algicola DG893	Marine broth, 28 °C	Laboratory culture of dinoflagellate *Gymnodinium catenatum* YC499B15	DSMZ	16394	[16] GenBank: EDM48791.1 UniProt accession number: A6EXA3
Oceanibulbus indolifex Hel45	Marine broth, 28 °C	Seawater	DSMZ	14862	[16] GenBank: EDQ04457.1 UniProt accession number: A9E8L0

Table 3.2. Characteristics of PHB depolymerases of the strains with demonstrated PhaZ activity.

SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Bacterial strain	Estimated molecular weight of the mature protein		pH range	Temperature range	GenBank	References
	From protein sequence [kDa]	Experimental [kDa]				
Comamonas testosteroni 31A	50.1	Gel filtration: 45 ± 2 SDS-PAGE: 44 ± 2	4.8 to 10.6; optimum 9.4	4 to 58 °C; optimum 29 to 35 °C	U16275.1	[20,21]
Cupriavidus sp.	46.86	Gel filtration: 48 SDS-PAGE: 50	4 to 10*; optimum 7.5		J04223.2	[17,22] [27]*
Paucimonas lemoignei	PhaZ1: 39.5 PhaZ2: 41.8 PhaZ3: 41.2 PhaZ4: 57. 5 PhaZ5: 42.2 PhaZ6: 41.0 PhaZ7: 36.2	PhaZ7: SDS-PAGE: 36 ± 2 Gel filtration: 36.2 ± 0.05				[8,23,28-30] Compiled in [31]
Pseudomonas stutzeri	57.51	SDS-PAGE: approx. 60	5 to 12; optimum 7.0		AB012225.1	[24,25]
Ralstonia sp.	Strain K1: 47.6 Strain A1: 47.4	SDS-PAGE: approx. 40 (strain K1) 49 (both strains) *	3 to 10; optimum 5.5 (strain K1)	12 to 50 °C; optimum 40 °C (strain K1)	D25315.1 AB022287.1 (Direct submission)	[26,27,32]

3.3.3. Growth conditions for pre-cultures

Pre-cultures in suspension were inoculated from colonies grown on agar plates of the corresponding recommended medium (Table 3.1, all media from BD Becton Dickinson except for R2A – DSMZ Medium 830). Incubation took place at the recommended temperature (Table 3.1) and 150 rpm until stationary phase was reached. Cell concentration

was monitored by measuring optical density of the cultures at 600 nm (OD_{600}) using a spectrophotometer (Biochrom, Ultrospec 50).

3.3.4. Bacterial degradation of PHB

Cultures were grown in 9 ml of modified mineral medium (Brunner, DSMZ Medium 457) containing 0.2 g of PHB films (cut into 1 cm^2 pieces). The composition of mineral medium was obtained from the DSMZ list of recommended media for microorganisms, and is as follows: Na_2HPO_4 2.44 g, KH_2PO_4 1.52 g, $(NH_4)_2SO_4$ 0.50 g, $MgSO_4 \bullet 7H_2O$ 0.20 g, $CaCl_2 \bullet 2H_2O$ 0.05 g, trace element solution SL-4 10 ml, distilled water 1000 ml (Trace element solution SL-4: EDTA 0.50 g, $FeSO_4 \bullet 7H_2O$ 0.20 g, trace element solution SL-6 100 ml, distilled water 900 ml. Trace element solution SL-6: $ZnSO_4 \bullet 7H_2O$ 0.10 g, $MnCl_2 \bullet 4H_2O$ 0.03 g, H_3BO_3 0.30 g, $CoCl_2 \bullet 6H_2O$ 0.20 g, $CuCl_2 \bullet 2H_2O$ 0.01 g, $NiCl_2 \bullet 6H_2O$ 0.02 g, $Na_2MoO_4 \bullet 2H_2O$ 0.03 g, 1000 ml). Phosphates were dissolved and autoclaved in a separate solution to avoid precipitation. Then, the two solutions were combined after they cooled down.

The films were previously weighed using an analytical balance (Denver Instruments, SI-234). Cultures were inoculated with 1 ml of pre-culture of the bacteria of interest and then incubated at 30 °C and 150 rpm for 7 days (Ecotron, Infors). PHB film degradation was evaluated by % mass loss at time points 6, 24, 48, 72, 96, and 168 h. For each time point, OD_{600} readings and cell counts — using a counting chamber (Double Neubauer Counting Chamber Set 3100, Hausser Scientific) and microscopy (Leica, DM RXA2) or plate dilutions — were performed. For the slow growing strains *M. algicola* and

O. indolifex, additional cultures were grown under the same conditions but with 1% w/v PHB film and left to grow for 2 weeks. All experiments were performed in triplicate.

For rapid determination of PHB degradation, double-layer mineral medium/agar plates were utilized (DSMZ Medium 474). The bottom layer consisted of 20 ml of mineral medium with agar (0.016 g/ml), and the top layer consisted of 10 ml of mineral medium with agar supplemented with 0.66 ml of sterile PHB suspension. The PHB suspension was prepared by stirring overnight and autoclaving at 121 °C for 5 minutes 0.03 g/ml of PHB powder (Sigma-Aldrich) in Milli-Q water. Samples of bacteria grown in pre-cultures or in PHB film-supplemented medium (1% w/v, 3 days, 30 °C), or of their extracellular fractions (see below), were spotted on the plates and incubated at 30 °C for at least one week.

3.3.5. Preparation of extracellular fractions

Extracellular fractions were obtained from cultures grown in mineral medium supplemented with 1% w/v PHB film (*C. testosteroni*, *Cupriavidus* sp., *P. lemoignei*, *P. stutzeri*, and *Ralstonia* sp.) for 3 days (30 °C, 150 rpm). These PHB-grown cultures were centrifuged (Sorval RC 6 PLUS, Thermo) at 10,000 × *g* and 4 °C for 30 minutes. The supernatants were recovered, filtered (0.2 μm syringe filter), and stored at 4 °C as extracellular fractions.

In some cases, extracellular fractions were also obtained from the PHB double layer agar plates. After incubating the cultures in solid media for several days, the agar was destroyed by adding mineral medium and passing it through a syringe (without needle). It was then incubated at 45 °C for 1 hour. The resulting samples were then centrifuged

(10,000 × *g* and 4 °C for 30 minutes) and filtered (0.2 µm syringe filter). The same procedure was carried out for plates without bacteria to be used as control.

3.3.6. PHB film mass loss experiments

PHB films were cut into 1 cm^2 pieces. Two pieces per sample to be tested were weighed using an analytical balance (Mettler Toledo, XS 105 Dual range) and exposed to 1 ml of extracellular fraction at 30 °C without agitation. The same procedure was carried out with 10-fold dilutions of the extracellular fraction. Samples were collected after 2, 6, 14, 24, and 48 h of exposure to the enzyme; the solutions were removed, and the films were rinsed two times with Milli-Q water; then, all liquid was removed, and the samples were left to dry at 50 °C for at least 2 days before the PHB films were weighed again. Control experiments were conducted with mineral medium only. All experiments were performed in triplicates.

3.3.7. Total protein quantification

Total protein concentration of the extracellular fractions was measured through fluorometric quantitation (Qubit 2.0, Life Technologies).

3.3.8. Extracellular fractions PHB plates assay

Double-layer mineral medium agar plates supplemented with PHB powder were pierced to produce cylindrical wells for the deposition of samples. For each plate, 100 µl of undiluted and 10-fold diluted extracellular fractions were dispensed in duplicate. Plates

were incubated at 4 °C, 15 °C, and 30 °C for 35 days and pictures were taken after 4 h, 3, 7, 14, and 35 days of incubation. The diameters of the halos formed by the degradation of the PHB were measured using ImageJ 1.46r (National Institutes of Health, USA), and the well diameters were subtracted.

3.3.9. Bioinformatics analysis

A protein functional analysis was done for each of the predicted PhaZs sequences using InterProScan — a tool that looks for predictive models (signatures) across several databases [33] — in order to confirm the classification from the PHA-DED [16]. Additionally, the amino acid sequence for each of the four predicted PhaZ was compared to the 12 other PhaZ sequences from the strains used in the present study through protein Basic Local Alignment Search Tool (BLASTp) analysis [34,35] to determine query cover and identities. The amino acid sequences for all of the selected PhaZs (demonstrated and predicted) were also compared by generating alignments with Multiple Alignment using Fast Fourier Transform (MAFFT) [36], Clustal Omega [37,38], and graphical representations with Geneious version 11.1.4 [39].

3.3.10. Statistical analysis

Two-tailed F-tests followed by the corresponding one or two-tailed t-test (two-sample equal variance, or two-sample unequal variance) were carried out to compare the statistical difference of data. The level of significance was set at $p < 0.05$.

3.4. Results

3.4.1. Microbial PHB degradation on plates

Initial tests were performed with the strains with demonstrated PhaZ activity (*C. testosteroni*, *Cupriavidus* sp., *P. lemoignei*, *P. stutzeri*, and *Ralstonia* sp.) by incubating cultures — previously grown in suspension at 30 °C with PHB as sole carbon source — deposited on PHB-mineral medium agar plates. As expected, these strains showed the formation of clear halos, indicative of PHB degradation (Figure 3.1, condition a). The cultures in suspension grew to a cell density on the order of 10^9 cells/ml, except for *P. lemoignei* which grew to 10^8 cells/ml. In order to only account for the extracellular PhaZ produced once the cultures were added to the agar plates, the cells were washed and resuspended in fresh medium before being deposited on the agar (Figure 3.1, condition b). The halo sizes were not significantly different between conditions a and b, except in the case of *P. stutzeri*, for which the halos were larger, and *Ralstonia* sp., for which they were smaller. Finally, in an attempt to directly compare the biodegradation potential of the strains tested, cell cultures spotted on the PHB plates were normalized to a cell density of 10^3 cells/ml (Figure 3.1, condition c). In all cases but for *Ralstonia* sp., the halos were noticeably smaller for this condition. In fact, *Ralstonia* sp. displayed the largest degradation areas for the three treatments after 7 days of incubation, followed by *C. testosteroni.*

By contrast, none of the strains with predicted PhaZ activity (*A. macleodii*, *L. vestfoldensis M. algicola*, and *O. indolifex*,) showed visible PHB degradation when plated, even when the plates were spotted from fully grown pre-cultures under recommended growth conditions (Table 3.1).

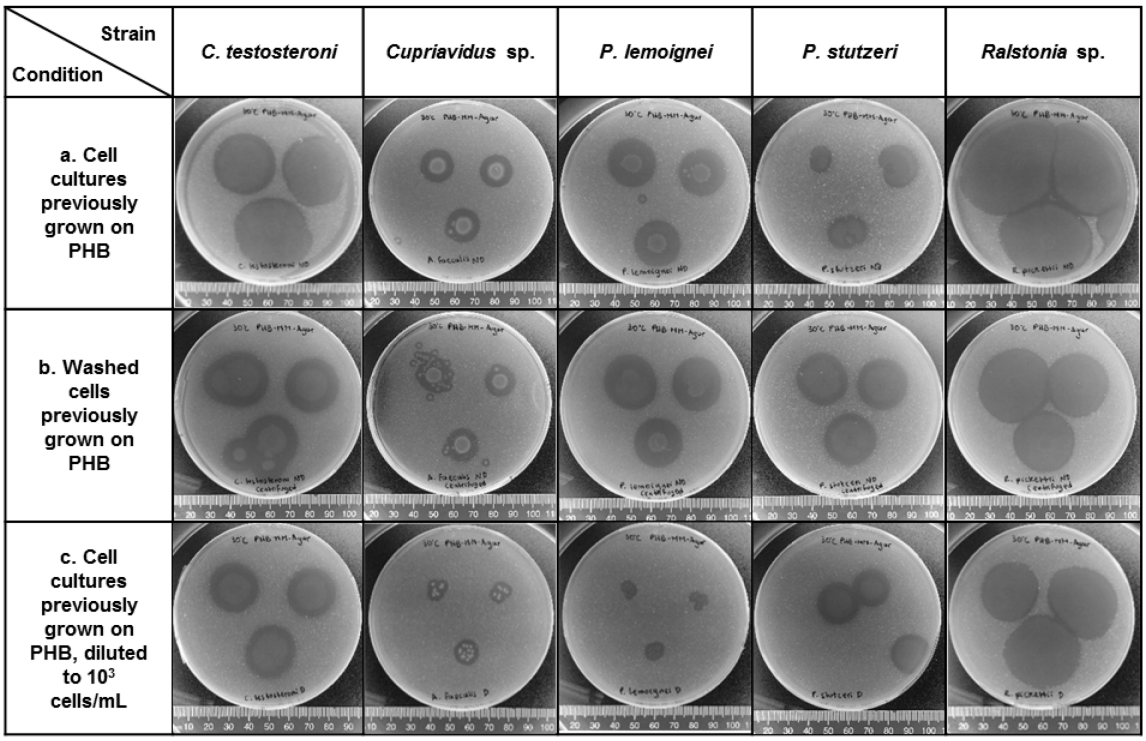

Figure 3.1. PHB degradation plate assay for bacterial cultures of strains with demonstrated PhaZ activity previously grown on PHB.

Incubation conditions: 3 × 10 µl of culture incubated for 7 days at 30 °C. Condition (a): spots of cell cultures previously grown in suspension on PHB; condition (b): spots of cells previously grown in suspension on PHB, washed by double centrifugation and resuspended in fresh medium; condition (c): spots of cell cultures previously grown in suspension on PHB and diluted to approximately 10^3 cells/ml. Halos indicate regions of visible PHB degradation.

3.4.2. PHB film exposed to bacterial cultures

To enable comparisons with other results from the literature (wherein PHB was processed by a different technique) the characteristics of PHB before (pellets) and after (film) dissolution in acetic acid were tested. Mw values for the PHB pellet and film were $2.48 \times 10^5 \pm 1 \times 10^3$ and $2.45 \times 10^5 \pm 1 \times 10^3$ g/mol, respectively, and the Mw/Mn were

3.10 ± 0.07 and 3.0 ± 0.1, respectively. The Mw and Mw/Mn values decreased slightly, which was found to be significant for Mw but not for Mw/ Mn (student's t-test analysis).

The degradation of PHB film was determined for all strains grown in suspension by assessment of PHB mass loss over time (Figure 3.2). Figure 3.2a shows that in the presence of *Ralstonia* sp. and *Cupriavidus* sp., the strains displaying the fastest degradation rates, approximately 90% mass loss was observed after only one week of incubation. *C. testosteroni* followed with a slower degradation rate — achieving 52.8% PHB film mass loss after one week. The degradation by *P. stutzeri* reached a plateau earlier, with around 15% mass loss after 24 h. Degradation with *P. lemoignei* was even slower as it reached a plateau of approximately 12% mass loss after 48 h.

No significant degradation of the PHB film was observed when exposed to *O. indolifex*, *A. macleodii*, or *L. vestfoldensis* (Figure 3.2b). In the case of *M. algicola,* however, the mass loss increased throughout the incubation period to reach approximately 5% after one week. Additional experiments were carried with *M. algicola* and *O. indolifex* to determine if longer exposure times (1% w/v PHB film exposed for 336 h) would lead to greater degradation. The results (Figure 3.2c) confirmed significant PHB degradation by *M. algicola* – mass loss of 11% –, while *O. indolifex* did not show degradation.

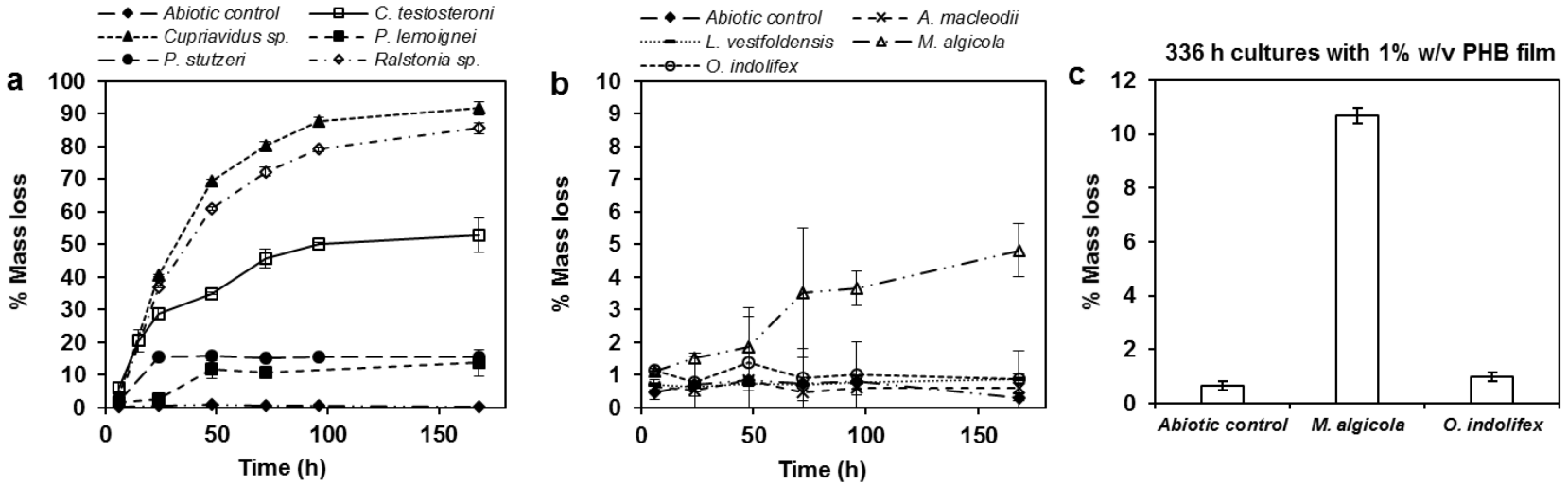

Figure 3.2. PHB film mass loss (%) for bacterial cultures grown in mineral medium at 30 °C and 150 rpm with PHB film as the sole carbon source.

(a) Strains with previously demonstrated PhaZ activity in the presence of 2% w/v PHB, and (b) strains with predicted PhaZ activity in the presence of 2% w/v PHB. (c) Degradation of PHB after 336h by *M. algicola* and *O. indoliflex* in the presence of 1% w/v PHB, otherwise in the same conditions.

Cultures grown with PHB films were monitored by OD_{600} — reflecting both bacterial growth and release of PHB particles from the film — and cell count, which can be observed in Figure 3.3a and b, respectively. Formation of biofilm on the PHB films was not evident from visual inspection. Differences in the initial values of OD_{600} and cells/ml between each strain can be seen and are due to different levels of growth in the pre-cultures. From 24 to 168 h, *Cupriavidus* sp. and *C. testosteroni* had the highest values for both OD_{600} (8.9 and 8.1, respectively) and cell density (reaching 6.5×10^9 and 5×10^9 cells/ml, respectively), followed to a lower degree by *Ralstonia* sp. (OD_{600} of 3.2 and 2.7×10^9 cells/ml). Low levels of growth (below OD_{600} of 0.5 and less than 5×10^8 cells/ml) were observed for the strains with predicted PhaZ activity. Additional controls were performed for the three strains with the highest OD_{600} values to corroborate that these values were not caused by remaining media from pre-cultures, or presence of trace amounts of acetic acid in the PHB films. In addition, thermogravimetric analysis performed on PHB films solvent cast with acetic acid by Anbukarasu et al. [18] showed no significant residual acetic acid was present in the films at the end of the procedure. No significant increases in OD_{600} measurements were obtained from these control experiments; these results can be observed in APPENDIX A, Figure A.1.

Figure 3.3c and d show the mass loss data normalized per OD unit and per cell, respectively, for the strains that displayed PHB degradation. In the initial stages of the culture (6 h), most strains showed slow growth and little degradation — consistent with a period of adaptation to the new carbon source. In contrast, *P. lemoignei* initially exhibited relatively high values of % PHB mass loss per OD_{600} (22% per OD_{600} unit) and % PHB mass loss per cell (6×10^{-8} % per cell), suggesting that *P. lemoignei* adapted to the initial

conditions the fastest. However, after the first six hours, other strains degraded more PHB than *P. lemoignei*. Specifically, *Ralstonia* sp. prevailed as the most efficient degrader (24% mass loss per OD_{600} and 3.3×10^{-8} % per cell, Figure 3.3c and d).

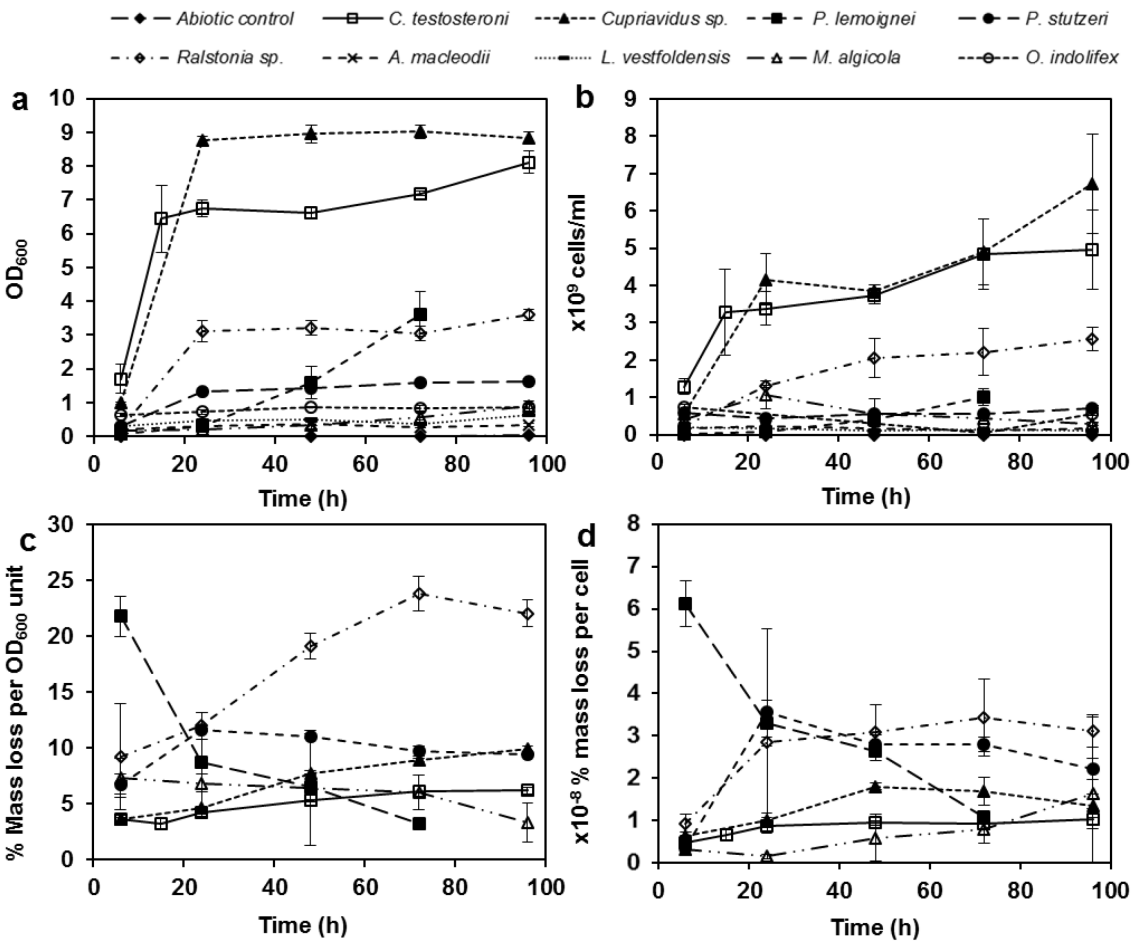

Figure 3.3. Growth and PHB film degradation by cultures in suspension.
(a) OD_{600}, (b) cell density, (c) % PHB mass loss per OD_{600}, (d) % PHB mass loss per cell.

Other parameters, indicative of activity, were also monitored during cultures. These included total protein concentration in the extracellular fraction (shown in Figure 3.4a for the five strains showing PHB degradation), and pH (shown in Figure 3.4b for *Cupriavidus*

sp., *P. stutzeri*, and *Ralstonia* sp.). The total protein concentration increased gradually for *C. testosteroni, Cupriavidus* sp., *Ralstonia* sp. and *P. lemoignei* (reaching 115, 92, 89 and 67 μg/ml, respectively), while a plateau at approximately 50 μg/ml was observed after 48 h for *P. stutzeri*. It can also be observed that the initial protein concentrations vary for all the samples — due to protein contents carried from the inocula and the starter culture media. In fact, the method used to obtain extracellular fractions from samples — a 10-fold dilution in mineral medium, followed by centrifugation and filtration — was performed with each medium alone to assess background protein contents. The resulting values were 61.9 ± 4 μg/ml for marine broth, 48.6 ± 4 μg/ml for tryptic soy broth, 39.8 ± 3 μg/ml for nutrient broth, 20.8 ± 2 μg/ml for R2A medium, and 12.8 ± 1 μg/ml for mineral medium alone). Taking this into account, the fact that *M. algicola* represented the only case where the protein concentration decreased after 6 h of incubation was likely due to the presence of the remaining marine broth in the inoculum; after this decrease, the protein concentration increased again. The concentration was also measured for the abiotic control to observe mineral media background, which remained low and constant throughout. Overall, these results are in general agreement with the trends obtained in the PHB film % mass loss (Figure 3.2a). In addition, for the bacteria that degrade the films most effectively (*Cupriavidus* sp. and *Ralstonia* sp.), as well as for *P. stutzeri,* pH decreased sharply to values below pH 5 after 24 h (Figure 3.4b).

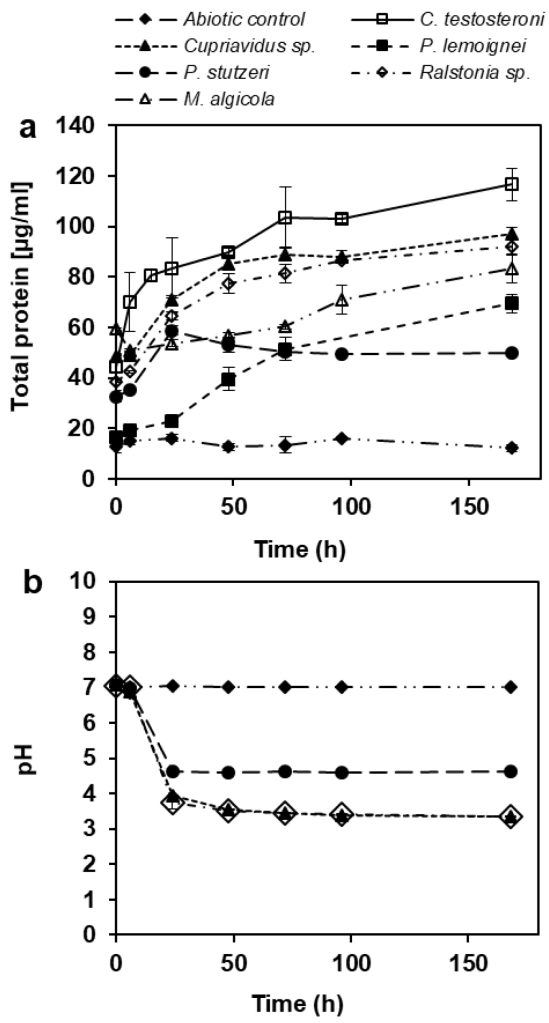

Figure 3.4. (a) Total protein concentration in the extracellular fraction and (b) pH for cultures grown in suspension using PHB film as sole carbon source.

3.4.3. PHB degradation by extracellular fractions

Since the PHB depolymerases of the bacterial strains with demonstrated PhaZ activity studied are excreted, and to decouple the impacts of enzymatic activity and bacterial growth on degradation, PHB films were exposed to extracellular fractions. These fractions were obtained from filtrates of cultures grown for 72 hours in suspension on 1%

w/v PHB film. Extracellular fractions were used either as is or diluted 10-fold with mineral medium.

Figure 3.5 displays the % mass loss of PHB film for non-diluted and diluted extracellular fractions. Fractions obtained from *Cupriavidus* sp. and *Ralstonia* sp. cultures showed the greatest PHB film % mass loss for both non-diluted (53% and 40%, respectively) and diluted extracts (70% and 58%, respectively); both results were statistically equal. These were followed by extracts from *C. testosteroni* (20% for the undiluted extract and 24% for the diluted extract). No significant degradation was observed for fractions from *P. leimoignei* and *P. stutzeri* after 48 h of exposure. The general trends of mass loss were similar for both the non-diluted and diluted extracts, however greater degradation was observed with diluted fractions. In fact, statistical analysis revealed that there was a significant increase in degradation at 6, 15, and 24 h when extracellular fractions of *Cupriavidus* sp. were diluted; at 15 and 24 h when fractions of *Ralstonia* sp. were diluted; and, interestingly, only at 2 h for diluted fractions of *C. testosteroni*.

An alternative method to obtain extracellular fraction from *P. stutzeri* was attempted by culturing the bacteria on PHB plates and extracting the enzymes present in the halos (which indicate hydrolysis of the polymer) by breaking and soaking the agar in a solution. After exposing PHB film to the resulting solution (pH 6.81) for 6 h at 30 °C, a mass loss of 13 ± 2% was reached (Figure 3.5c). These results are comparable to the values achieved after 6 h of exposure to the diluted extracellular fractions of *Cupriavidus* sp. and *Ralstonia* sp. (Figure 3.5b), which were 12.5 and 13.5% respectively.

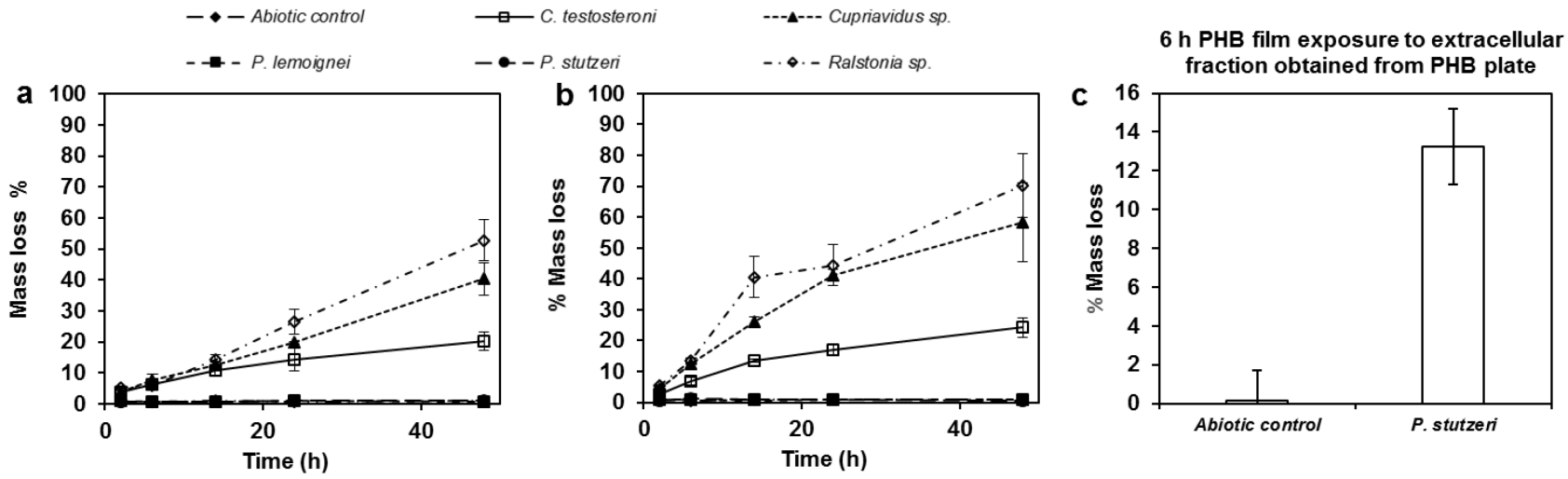

Figure 3.5. % PHB mass loss after exposure to extracellular fractions.

(a) Crude extracellular fractions obtained from liquid cultures, and (b) extracellular fractions diluted 10-fold in mineral medium at 30 °C. Curves for extracellular fractions from *P. lemoignei*, *P. stutzeri*, and the abiotic control overlap. (c) % mass loss after 6 h of exposure to control and to extracellular fraction of *P. stutzeri* obtained from fractionated PHB plates instead of liquid cultures.

It is important to note that, when comparing these results with degradation by cultures, the period allowed for degradation was shorter (48 h for the extracts compared to 168 h for the cultures). Moreover, these results do not account for the possible different levels of PhaZ expression in the various strains studied, which would affect both the % mass loss and the degradation rate.

3.4.4. Extracellular fractions PHB plates assay

The deposition of extracellular fraction samples into cylindrical wells in PHB-agar plates resulted in translucent halos corresponding to zones in which PHB was degraded. Figure 3.6 shows the evolution of the diameters of these halos for non-diluted extracellular fractions of *C. testosteroni*, *Cupriavidus* sp., and *Ralstonia* sp. incubated at 4 °C, 15 °C, and 30 °C. As expected, faster degradation was observed at greater temperatures. The best performance was seen for the fractions incubated at 30 °C (31.7 mm, 48,7 mm and 41.1 mm halo diameter, respectively for each strain), and the worst for the fractions incubated at 4 °C (4.3 mm, 13.4 mm and 13.9 mm halo diameter, respectively).

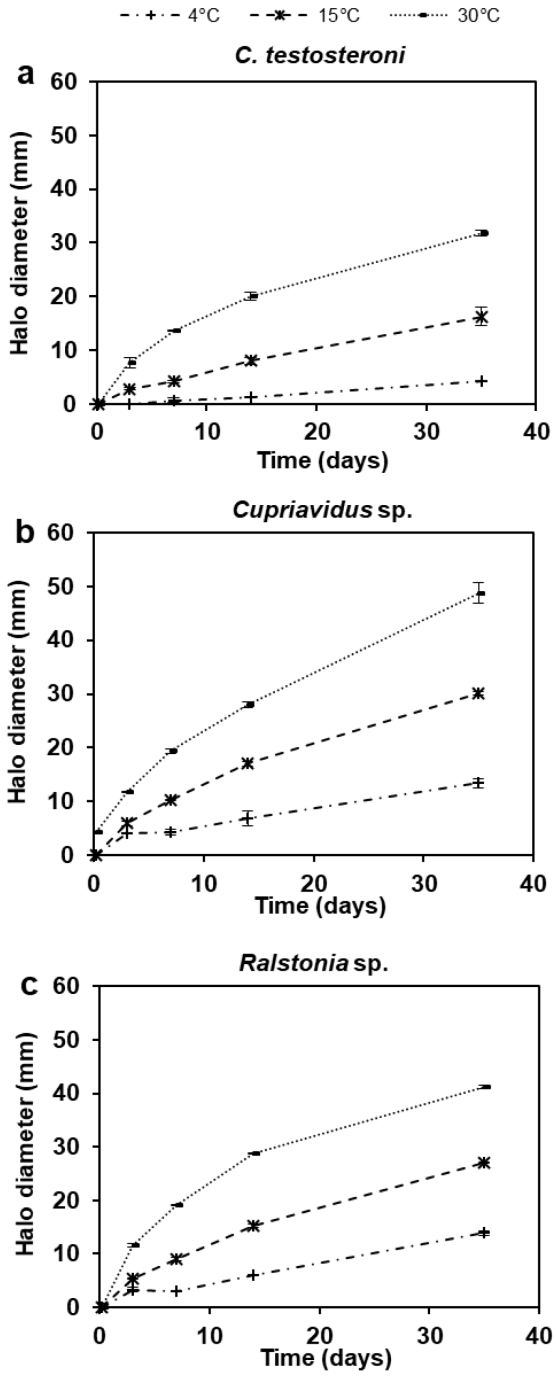

Figure 3.6. Evolution of diameter of halos on PHB-agar plates resulting from the degradation of PHB by non-diluted extracellular fractions.

Extracellular fractions from (a) *C. testosteroni*, (b) *Cupriavidus* sp., and (c) *Ralstonia* sp. cultures, as a function of time. Results are shown for PHB-agar plates incubated at 4 °C, 15 °C, and 30 °C

3.4.5. Bioinformatics analysis

Table 3.3 contains the main results from the protein functional analysis performed with InterProScan for each of the 4 predicted PhaZs studied. Individual analyses were also ran for each sequence on the databases Pfam [40], SignalP 4.1 Server [41], and Phobious [42] to corroborate the InterProScan results (only slight differences in the amino acids ranges were found for the Pfam families and domains, but the signatures were the same). All of the predicted sequences were classified as extracellular PHB depolymerases; but it should be noted that neither the SignalP 4.1 nor the Phobious analyses identified the presence of a signal peptide (SP) in the *L. vestfoldensis* and *O. indolifex* sequences.

Table 3.3. InterProScan analysis.

Number ranges indicate amino acid range where the signature was identified. InterPro member databases are indicated in bold.

PhaZ source bacterial strain (GenBank reference, amino acids length)	InterProScan analysis					
	Family	**Homologous superfamilies**	**Domains**	**Signal peptide prediction**	**Gene Ontology (GO) term prediction**	**Other signatures**
Alteromonas macleodii (AFS36858.1, 356)	Esterase, PHB depolymerase (IPR010126) Details: 246 - 341: **Pfam** PF10503 (Esterase_phd)	22 - 342: Alpha/Beta hydrolase fold (IPR029058) Details: 39 - 342: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 22 - 182: **GENE3D** (G3DSA:3.40.50.1820) 223 - 339: **GENE3D** (G3DSA:3.40.50.1820)	41 - 193: Peptidase S9, prolyl oligopeptidase, catalytic domain (IPR001375) Details: 41 - 193: **Pfam** PF00326 (Peptidase_S9)	1 - 28: **PHOBIUS** SIGNAL_PEPTIDE (Signal Peptide) 1 - 28: **SignalP** Gram- prok SignalP-noTM (SignalP-noTM)	Biological Process: GO:0006508 proteolysis Molecular Function: GO:0008236 serine-type peptidase activity GO:0016787 hydrolase activity Cellular Component: GO:0005576 extracellular region	98 - 118: **COILS** Coil (Coil) 29 - 356: **PHOBIUS** NON_CYTOPLASMIC_DOMAIN (Non cytoplasmic domain) 29 - 341: **PANTHER** PTHR42972 (FAMILY NOT NAMED) 29 - 341: **PANTHER** PTHR42972:SF2 (SUBFAMILY NOT NAMED)
Loktanella vestfoldensis (EAQ05513.1, 303)	Esterase, PHB depolymerase (IPR010126) Details: 28 - 238: **TIGRFAMs** TIGR01840 (TIGR01840) 28 - 222: **Pfam** PF10503 (Esterase_phd)	3 - 300: Alpha/Beta hydrolase fold (IPR029058) Details: 24 - 114: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 111 - 300: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 3 - 271: **GENE3D** (G3DSA:3.40.50.1820)	None predicted	None predicted	Molecular Function: GO:0016787 hydrolase activity Cellular Component: GO:0005576 extracellular region	25 - 296: **PANTHER** PTHR43037 (FAMILY NOT NAMED)

Table 3.3. (cont.).

PhaZ source bacterial strain (GenBank reference, amino acids length)	InterProScan analysis					
	Family	**Homologous superfamilies**	**Domains**	**Signal peptide prediction**	**Gene Ontology (GO) term prediction**	**Other signatures**
Marinobacter algicola DG893 (EDM48791.1, 580)	Esterase, PHB depolymerase (IPR010126) Details: 53 - 263: **TIGRFAMs** TIGR01840 (TIGR01840) 53 - 248: **Pfam** PF10503 (Esterase_phd)	27 - 359: Alpha/Beta hydrolase fold (IPR029058) Details: 27 - 245: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 29 - 359: **GENE3D** (G3DSA:3.40.50.1820) 361 - 437: Immunoglobulin-like fold (IPR013783) Details: 361 - 437: **GENE3D** (G3DSA:2.60.40.10)	364 - 434: Domain of unknown function DUF5011 (IPR032179) Details: 364 - 434 **Pfam** PF16403 (DUF5011)	1 - 32: **PHOBIUS** SIGNAL_PEPTIDE (Signal Peptide) 1 - 32: **SignalP** Gram- prok SignalP-noTM (SignalP-noTM)	Molecular Function: GO:0016787 hydrolase activity Cellular Component: GO:0005576 extracellular region	33 - 580: **PHOBIUS** NON_CYTOPLASMIC_DOMAIN (Non cytoplasmic domain) 44 - 353: **PANTHER** PTHR43037 (FAMILY NOT NAMED) 13 - 35: **TMHMM** TMhelix (TMhelix)
Oceanibulbus indolifex Hel45 (EDQ04457.1, 357)	Esterase, PHB depolymerase (IPR010126) Details: 95 - 281: **TIGRFAMs** TIGR01840 (TIGR01840) 93 - 278: **Pfam** PF10503 (Esterase_phd)	62 - 354: Alpha/Beta hydrolase fold (IPR029058) Details: 73 - 180: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 178 - 282: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 310 - 354: **SUPERFAMILY** SSF53474 (alpha/beta-Hydrolases) 62 - 352: **GENE3D** (G3DSA:3.40.50.1820)	None predicted	None predicted	Molecular Function: GO:0016787 hydrolase activity Cellular Component: GO:0005576 extracellular region	44 - 353: **PANTHER** PTHR43037 (FAMILY NOT NAMED)

Figure 3.7 shows the results for the global alignment, identities, and the consensus sequences of specific regions of the amino acid sequences of all 13 proven and potential PhaZs investigated in the present study based on MAFTT analysis.

Figure 3.7a shows that most PhaZ amino acid sequences analyzed displayed a good alignment with high levels of consensus around an oxyanion histidine (H) residue and the serine (S), aspartic acid (D) and H residues of the catalytic triad of the catalytic domain (Cat), and in the substrate binding domain (SBD) region [31]. However, a few exceptions are important to note. Firstly, the sequences for PhaZ from *O. indolfex* and *L. vestfoldensis* did not align with the SBD region. Secondly, the sequences from *A. macleodii* and PhaZ7 from *P. lemoignei* poorly aligned with the SBD and did not cover the full region. Thirdly, the alignment was not as strong around the H oxyanion of Cat for the sequences of PhaZ from *A. macleodii* and *C. testosteroni* compared to the other PhaZ sequences tested.

Figure 3.7b shows the comparison of amino acid identities between all 13 PhaZ amino acid sequences investigated. High levels of similarity were found for PhaZs from *M. algicola* and *P. stutzeri* (67% identity), PhaZ2 and PhaZ3 from *P. lemoignei* (70% identity), PhaZs from *Cupriavidus* sp. and *Ralstonia* sp. (83% identity), and the latter two with PhaZ5 from *P. lemoignei* (58% and 59% identity, respectively). It should also be noted that PhaZ7 from *P. lemoignei* and PhaZ from *A. macleodii* displayed identities below 15% for practically all comparisons.

More in-depth analysis of the alignment of residues close to the key amino acids of the catalytic domain (oxyanion H, and catalytic triad amino acids — S, D, and H) is shown in Figure 3.7c. All sequences analyzed conserved these four key residues except for *A. macleodii* and *C. testosteroni*, which did not conserve the H oxyanion, and for PhaZ7 from

P. lemoignei which did not conserve the D and H residues of the catalytic triad. Otherwise, most of the sequences showed strong alignment around the H oxyanion and the S residue of the catalytic triad. More variation was observed around D and, to a greater extent, the H residue of the catalytic triad. PhaZ7 showed the least agreement with the consensus sequence.

Figure 3.7d shows a similar analysis for a section of the approximate location of the SBD. In this case, the predicted sequences of *O. indolifex* and *L. vestfoldensis* did not align with the SBD sequences of other PhaZs. Moreover, PhaZ7 from *P. lemoignei*, and PhaZ from *A. macleodii* aligned very poorly, with their sequences ending prematurely, perhaps indicative of incomplete domains.

A similar comparative amino acid sequence analysis was performed using Clustal Omega, which can be found in APPENDIX A, yielded similar results for global alignment, relative identities, and amino acid alignment in Cat and SBD (Figure A.2).

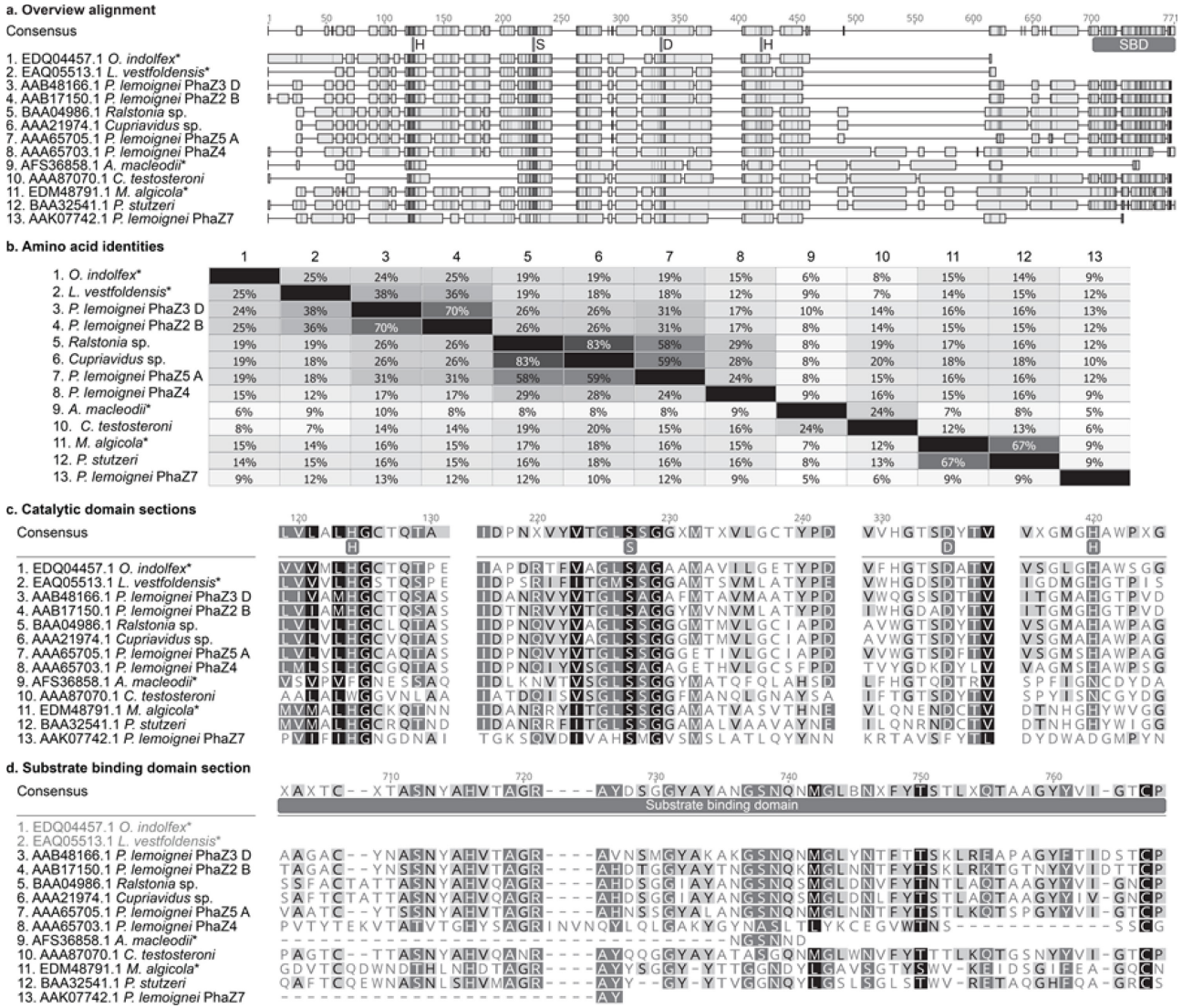

Figure 3.7. Comparison of the amino acid sequences of demonstrated and predicted (*) PhaZs by MAFFT alignment.

(a) Global alignment overview, (b) identities heat map, (c) main sections of the catalytic domain, and (d) part of the estimated location of the substrate binding domain (SBD). Identities are indicated by darker shaded amino acids. Annotations are located below the consensus sequence: oxyanion histidine (first H), catalytic triad amino acids (serine (S), aspartatic acid (D), and second H), and part of the SBD.

3.5. Discussion

Most studies involving PHB degradation by PhaZ-producing microorganisms have focused on screening strains or on evaluating the effect of an isolated enzyme on bulk PHB. In fact, few reports have performed extensive comparisons of the interaction between extracellular PhaZ-producing microorganisms and PHB films; notable exceptions include studies performed by Hsu et al. [43], where the authors purified and determined characteristics of the investigated PhaZ from *Streptomyces bangladeshensis* 77T-4, and by Wang et al. [44], whose research included PHB film characterization after degradation from the strain *Pseudomonas mendocina* DS04-T. Most of those studies have investigated PHB degradation by a given bacterial strain under a given set of conditions (temperature, medium, PHB treatment and surfaces, etc.), making it difficult to compare degradation rates between species or strains. One of the aims of the current study is to establish a clear assessment of PHB film degradation between strains, using well-defined experimental conditions.

In the present study, several pieces of PHB film (rather than single or very few films) were introduced into the media as sole carbon source (with an available surface area of approximately 62 cm^2) to improve PHB availability in the culture. The degradation experiments conducted with *Cupriavidus* sp., *Ralstonia* sp., and *C. testosteroni* displayed trends consistent with Langmuir kinetics (Figure 3.2a) — as would be expected for a reaction linked to surface adsorption and growth-associated enzymatic product such as PhaZ. Likewise, the slow increase in mass loss seen with *M. algicola* (Figure 3.2b) is also consistent with the early stages of Langmuir kinetics. However, this was not the case for

degradation in the presence of *P. lemoignei* (Figure 3.2a), which showed almost no mass loss for the first 24 h followed by degradation increase up to 48 h, to finally reach a plateau at 12% mass loss. A possible explanation for the lower degradation from *P. leimoignei* is that the culture conditions were not optimal for PhaZs production by this strain. It has been observed that PhaZ synthesis can be induced by the presence of the polymer, but can also take place in its absence [3,31]; for instance, *P. leimoignei* has a maximum PhaZ production when it is grown with succinate as carbon source under alkaline conditions, due to starvation caused by poor succinate transport into the bacteria above pH 7 [31].

When comparing the degradation observed herein to that obtained in previous studies, it is important to note that the PHB film used in this investigation was produced using a novel fabrication method based on acetic acid solvent casting [18], rather than classical chloroform-based methods. One advantage of this approach is the elimination of residual chloroform, which could potentially have a negative impact on cells. This fabrication method confers the films with different properties influencing biodegradation, such as degree of crystallinity [18]. To determine whether processing with acetic acid could affect the molecular weight of the polymer, we conducted GPC experiments on initial pellets and on pellets dissolved in acetic acid at 118 °C for 8 hours. The average polydispersity did not change significantly, and the weight-average molecular weight only decreased to a small extent. According to the mechanism of action of most PhaZs, the enzyme first binds to the crystalline regions of the polymer before hydrolyzing polymer chains in the amorphous regions; this is followed by significantly slower degradation of the crystalline region [45]. Therefore, the degradation of PHB films with lower crystallinity is generally favoured. The film used for degradation by *P. mendocina* DS04-T had a

reported crystallinity of 47.35% [44], while the crystallinity of the films used in the present study was approximately 70% as measured by X-ray diffraction (between 5° and 60°) [18]. This highlights the need for more standardized and systematic study of PHB degradation when different PhaZs are investigated, and the impact of PHB processing and products on this degradation.

Of the strains with predicted PhaZ, *M. algicola* was able to yield PHB films mass loss in liquid cultures (Figure 3.2b), even if no clear degradation was observed on PHB-agar plates. It is possible that the lack of degradation on the plate was caused by low PhaZ expression and mass transfer limitations (increase in path length for the diffusion of the enzyme caused by gel structure through characteristics impeding the degradation halo effect or the growth of colonies, such as porosity and tortuosity [46]). *M. algicola* DG893, isolated from cultures of the dinoflagellate *Gymnodinium catenatum* YC499B15, was first reported by Green et al. [47] and was predicted to produce extracellular PhaZ type I belonging to the homologous family 9 [16]. Green et al. showed that the bacteria could use the PHB monomers α-hydroxybutyrate and β-hydroxybutyrate as sole carbon sources but did not demonstrate PHB degradation. The present study shows that *M. algicola* is able to cause PHB films mass loss, but further studies are necessary to support that this strain is an extracellular PHB degrading species.

The amino acid sequence of PhaZ form *M. algicola* shows all the features of an extracellular PhaZ, with predicted SP (Table 3.3) and an apparent good alignment in Cat and SBD (Figure 3.7). The high level of identity of this amino acid sequence with PhaZ from *P. stutzeri* confirmed by three different analyses (Figure 3.7b, Figure A.2b, and Table A.1) agrees with its classification found in the PHA-DED (both sequences classified under

the homologous family e-dPHAscl depolymerases (type 1) homologous family 9 [16]), and supports, along with its utilization of PHB for growth (Figure 3.2b), the hypothesis that *M. algicola* could produce an active extracellular PhaZ. Looking at the similarities between these two sequences in more detail, the global alignment (Figure 3.7a) shows very similar patterns, including gap generation. When focusing on the alignment around the key residues of Cat, only 9 differences out of 60 amino acids are observed (Figure 3.7c), and 26 differences out of 58 aligned residues are found in the region of the SBD investigated (Figure 3.7d). These differences, and possibly others found in the whole sequences, likely affect folding, binding to PHB and/or the reactivity of Cat which lead to *P. stutzeri* degrading the polymer faster than *M. algicola* under the experimental conditions tested (Figure 3.2a and b).

After performing a Basic Local Alignment Search Tool (BLAST) [34] analysis for PhaZ from *M. algicola* on UniProt (accession number: A6EXA3) and the National Center for Biotechnology Information (NCBI) (GenBank: EDM48791.1) databases, high similarities (97 – 75%) with other predicted enzymes from strains of the genus *Marinobacter* were found. Among these, only *Marinobacter* sp. NK-1 has been demonstrated to degrade PHB, and a recombinant version of its PhaZ has been produced and characterized [48,49]. A discontiguous megablast nucleotide alignment between the *Marinobacter* sp. NK-1 *phaZ* gene sequence (GenBank: AB079799.1) and *M. algicola*'s predicted *phaZ* region (GenBank: ABCP01000004.1, Region: 46935 – 48677) showed 73% identity (1214/1656) between positions 1042 and 2680 for *Marinobacter* sp. NK-1 and positions 48585 and 46941 for *M. algicola*, covering most of the mature peptide nucleotide sequence (Figure A.4). A BLASTp alignment (between GenBank:

EDM48791.1 for *M. algicola* and GenBank: BAC15574.1 for *Marinobacter* sp. NK-1) showed 75% identity and 85% positives (taking into account similar residues) between the amino acid sequences Figure A.5). Analysis of the *phaZ* gene sequence from *Marinobacter* sp. NK-1 suggests it belongs to the same homologous family as *phaZ* from *M. algicola* DG893 (see database from Knoll et al. [16], NCBI accession code protein sequences BAC15574.1 and 22779267 come from the nucleotide sequence under the GenBank number AB079799.1). Further investigation of this strain is described in Chapter 5.

On the other hand, the rest of the strains with predicted PhaZ investigated (*A. macleodii*, *L. vestfoldensis*, and *O. indolifex*) showed no significant growth or PHB degradation under the conditions tested (Figure 3.2b, Figure 3.3a). When considering the key amino acids of Cat, successful alignments for most predicted sequences (Figure 3.7c), confirm the InterProScan signatures classification (Table 3.3) towards extracellular PhaZs. However, the fact that no SPs in the sequences from *L. vestfoldensis* and *O. indolifex* — as determined using multiple analytical approaches (Table 3.3, Figure 3.7, Figure A.2) — could explain why these predicted enzymes did not show PhaZ activity. The SP of PhaZ is essential for enzymatic secretion [31] and its absence in the protein sequences from *L. vestfoldensis* and *O. indolifex* (GenBank EAQ05513.1 and EDQ04457.1 respectively) is likely indicative of wrongful classifications as extracellular PhaZs or a potential loss of function. The former case has been previously reported for a sequence belonging to *Rhodospirillum rubrum* which was initially identified as an extracellular PhaZ in the PHA-DED but was later reclassified as an intracellular PhaZ (i-PhaZ) following further analyses and experimental data [50]. In addition, upon analysis of sequences from *Azotobacter vinelandii* and *Beijerinckia indica* predicted to code for extracellular PhaZs but missing

sequences for SPs and considering that no extracellular PhaZ activity was found in cultures of these strains, Sznajder and Jendrossek concluded that the classification of intracellular and extracellular PhaZs found in the PHA-DED was not always reliable. An InterProScan analysis of the sequence of *R. rubrum* (data not shown) displayed similar features to sequences of *L. vestfoldensis* and *O. indolifex* (Table 3.3) — including extracellular region for the gene ontology of the cellular component — and found it to fall under the same e-dPHAscl depolymerases (type 1) homologous family 2 as the latter sequence. This suggests i-PhaZs may have some general similarities to extracellular PhaZs at the global protein level, but that specific features, such as SP and sequences in Cat and SBD (Figure 3.7) display significant variability. In the case of *A. macleodii,* for which an SP sequence is identified (Table 3.3), it may be possible for this strain to elicit extracellular PhaZ activity under different culture conditions.

Another important factor involved in the lack of PhaZ activity of *O. indolifex*, *L. vestfoldensis*, and *A. macleodii* was their lack or very poor alignment to the SBD sequences. The sequences from these strains lack the conserved histidine, arginine, and cysteine residues found in both types of SBDs found in most extracellular PhaZs [31]. A quick MAFFT alignment of the predicted sequences of this study to the demonstrated i-PhaZ from *R. rubrum* [50] (GenBank: ABC22769.1) shows a general good alignment for *O. indolifex* and *L. vestfoldensis* and identities of 42% and 21% correspondingly to the sequence of *R. rubrum* (Figure A.3). On the other hand, PhaZ sequences from *M. algicola* and *A. macleodii* had poorer alignments and identities of 20% and 10%, respectively. This short exploration towards intracellular PhaZs suggests the predicted sequences which were found non-active could be coding for i-PhaZs.

The sequence of PhaZ7 from *P. lemoignei* differed in many ways from the other PhaZ sequences investigated: it showed low identity (in many cases below 10%) with all other sequences (Figure 3.7b), showed the most variation in the areas of interest of the catalytic domain (Figure 3.7c), and had practically no alignment to the SBD sequences, effectively not covering the whole region. This is not completely unexpected as this extracellular PhaZ is in many ways atypical: unlike most PhaZs, it has a different degradation mechanism and can readily degrade completely amorphous PHB [8].

Among the strains that showed the highest PHB degradation rates (*C. testosteroni*, *Cupriavidus* sp., and *Ralstonia* sp.), *Ralstonia* sp. displayed the highest specific degradation activity as measured per OD_{600} unit and per cell (Figure 3.3c and d). In fact, under the conditions tested, less biomass was required for this bacterium to degrade PHB compared to faster growing *Cupriavidus* sp. and *C. testosteroni* (Figure 3.3b). Typically, faster growing bacteria would be expected to excrete more PhaZ, further increasing the extracellular protein concentration, accelerating PHB degradation to monomers, providing more readily available substrate, and further accelerating growth. However, in the case of *Ralstonia* sp., while the biomass contents were lower than for fast growing species, the total amount of extracellular proteins was of the same order as that observed in the other strains (Figure 3.4a), suggesting a high level of protein excretion.

In the case of *P. stutzeri,* PHB mass loss (Figure 3.2a), growth (Figure 3.3a and b), and extracellular protein production (Figure 3.4a) all ceased to increase after 24 h. As can be seen in Figure 3.4b, this also corresponded to the pH of the culture reaching a plateau at pH 4.6. The decrease in pH in the tested cultures was due to the common release of metabolic by-products and the production of 3-hydroxybutyric acid (3HB) from PHB

degradation. To test if the acidity of the medium was impeding the growth of *P. stutzeri* and the activity of its PhaZ, cells from cultures grown on 1% w/v PHB film for 72 h (pH 4.8) were washed (centrifuged and resuspended in fresh medium) prior to plating on PHB-agar plates. These cells exhibited larger PHB degradation areas (Figure 3.1b) than the non-washed *P. stutzeri* (Figure 3.1a). This suggests that the PhaZ from *P. stutzeri* is strongly affected by pH below pH 5. According to data obtained by Uefuji et al. [25], the optimum pH values for this enzyme ranged between pH 7.0 and 7.5, and its activity decreased dramatically at pH 4.5 (from a maximum activity of 0.17 to 0.03 units/ml). Interestingly, this study also showed that, when resuspending the enzyme in solution at pH 7.4 and 37 °C after exposure for 5 hours over a wide range of pH values, the enzyme remained stable only when it was exposed to pH values between 6 and 12 [25]. Therefore, a pH drop to pH 4.6 in the case of the bacterial cultures (Figure 3.2a) and to pH 4.8 in the case of the extracellular fractions (Figure 3.5a and b) likely irreversibly inactivated the enzyme — even when the extracellular fraction was diluted 10-fold and the pH was re-established to a value of 6.9. In the case of the bacterial culture, this situation leads to carbon source limitation (less soluble products released from PHB film) and therefore to a standstill state in protein production (Figure 3.4a) and very slow cell growth (Figure 3.2a). The inactivation of the enzyme at low pH was also observed when evaluating the extracellular fractions with PHB film (Figure 3.5a and b). Furthermore, when excreted PhaZ from *P. stutzeri* was isolated from a PHB-agar plate at pH 6.81 (alternative method to obtain extracellular fractions, Figure 3.5c), the enzyme activity was comparable to that seen for the diluted fractions of PhaZ from *Cupriavidus* sp. and *Ralstonia* sp. (Figure 3.5b), since

in this case the enzyme from *P. stutzeri* was able to remain active through the whole process.

Unlike *P. stutzeri,* in the cases of degradation by *Cupriavidus* sp. and *Ralstonia* sp., PHB continued to be degraded at lower pH levels (down to a pH value of approximately 3.3) (Figure 3.3 and Figure 3.4b). This suggests the PhaZs from these strains are active over a wider pH range. In the case of *Ralstonia* sp., the optimal activity of the enzyme was found to be at pH ranging between 5.0 and 6.0, conserving activity even at pH 3.0 [26]. PhaZ from *Cupriavidus* sp. remains stable at pH values from 5.0 to 8.0, still retaining activity at pH 3.0 [51]. Given these observations on how low pH affects different strains and their PhaZ production, it can be said that the initial concentration of PHB available in bacterial cultures can be a key factor affecting PhaZ production due to the release of 3HB from PHB degradation. For instance, it has been reported that for *Streptomyces bangladeshensis* 77T-4, PhaZ production was good at an emulsified PHB concentration of 0.025% but that a decrease in degradation occurred when it was increased to 0.05 or 0.1% [43]. Therefore, it is possible that every strain has its own optimal initial PHB concentration (and even PHB absence should be considered for cases such as *P. lemoignei* [31]). It is also important to note that, since in this study PHB film was used instead of PHB powder, even though the PHB mass concentration was greater (0.2% w/v), there was less surface area available per mass for films compared to polymer particles.

In order to decouple the effect of bacterial growth from enzymatic activity, PHB degradation was investigated with extracellular fractions, crude as well as diluted 10-fold. The dilution was included to evaluate potential concentration inhibition as described by Mukai et al. [52] for PhaZs of *Cupriavidus* sp., *Ralstonia* sp., and by Uefuji et al. [25] for

PhaZ of *P. stutzeri.* The fact that PHB degradation remained constant or improved after dilution for the active extracellular fractions tested (Figure 3.5) does indeed suggest that concentration inhibition was significant in the crude extract.

Kinetic experiments (Figure 3.6) showed that depolymerisation rates decreased with low temperatures, which agrees with the reported optimal working conditions for PhaZs in general — which range between 29 and 70 °C [6]. Particularly, PhaZ from *C. testosteroni* is active between 4 and 58 °C but has an optimal range from 29 to 35 °C [20]; while PhaZ from *Ralstonia* sp. has an optimum temperature of 40 °C and denatures at 45 °C [26]. PhaZ from *Cupriavidus* sp. remains stable if it is incubated at temperatures up to 40 °C for half an hour, and the highest degradation rates are observed at 37 °C [51]. Our experiments showed that all extracellular fractions were still active at 4 °C, especially those from *Cupriavidus* sp. and *Ralstonia* sp.*,* for which degradation could be observed after only 3 days of incubation at this temperature (Figure 3.6a).

For future studies of specific PhaZ enzymes from the strains with demonstrated PhaZ activity, we summarized protein sizes and other characteristics in Table 3.2. Inconsistencies in the literature and changes in taxonomy over the years presented challenges for the direct identification of the PhaZs of interest. Such confusion could result from the fact that that the genera *Alcaligenes*, *Cupriavidus*, and *Ralstonia* seem to share several characteristics [53]. PCR analyses (Figure A.6) were performed. The analyses and protein identification are presented in APPENDIX A, section A.4 and helped clarify that PhaZs belonging to *Cupriavidus* sp. and *Ralstonia* sp. correspond to the sequences reported in Genbak J04223.2 for the first, and Genbank D25315.1 and AB022287.1 for the latter.

3.6. Conclusion

As PHB usage shows increasing promise for commercial applications based on its biocompatibility and biodegradation, such as food packaging or biosensors, available studies on PhaZs are gaining more value, and can serve as tools to further drive these developments, especially from a product life cycle perspective. However, the absence of direct comparisons between degrading microorganisms and enzymes presents a limiting factor. In this study, screening of strains with proven and predicted PhaZ activity for PHB film degradation helped identify which strains display high degradation activity under various sets of conditions. Analyses of PHB degradation by extracellular fractions also provided valuable information on the comparative PHB degradation potential by PhaZ enzymes. Producing, comparing, and analyzing recombinant versions of PhaZs from *C. testosteroni, Cupriavidus* sp.*, P. stutzeri,* and *Ralstonia* sp. can further clarify experiments conducted with extracellular fractions. These results can also potentially be used in the establishment of better models for PHB degradation mechanisms. Additionally, we showed that *M. algicola* DG893 is able to cause PHB films mass loss in liquid culture, which could point towards this strain being able to produce an extracellular PhaZ. And although the study of isolated strain cultures and enzymes cannot predict exactly how PHB will decompose in the environment with so many factors that come into play [3], our results can provide a starting point for the evaluation of mechanisms and the assessment of the fate of PHB under certain conditions.

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3.8. References

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4. Streamlined Production, Purification, and Comparison of Recombinant Extracellular Polyhydroxybutyrate Depolymerases[†]

4.1. Abstract

Heterologous production of extracellular polyhydroxybutyrate (PHB) depolymerases (PhaZs) has been of interest for around 30 years, but implementation is sometimes difficult and can limit the scope of research. With the constant development of tools to improve recombinant protein production in *Escherichia coli*, we propose a method that takes characteristics of PhaZs from different bacterial strains into account. Recombinant His-tagged versions of PhaZs (rPhaZ) from *Comamonas testosteroni* 31A, *Cupriavidus* sp.*, Marinobacter algicola* DG893, *Pseudomonas stutzeri,* and *Ralstonia* sp. were successfully produced with varying expression, solubility, and purity levels. PhaZs from *C. testosteroni* and *P. stutzeri* were more amenable to heterologous expression in all aspects; however, strategies were developed to circumvent low expression and purity for the other PhaZs. Degradation activity of the rPhaZs was compared using a simple PHB plate-based method, adapted to test for various pH and temperatures. rPhaZ from *M. algicola* presented the highest activity at 15 °C, and rPhaZs from *Cupriavidus* sp. and *Ralstonia* sp. had the highest activity at pH 5.4. The methods proposed herein can be used

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to test the production of recombinant soluble PhaZs, and to perform initial PhaZ evaluation for applications that require PHB degradation.

Keywords: *Escherichia coli*, extracellular PHB depolymerases (PhaZs), poly(3-hydroxybutyrate) (PHB), polymer degradation activity, recombinant expression.

4.2. Introduction

The study of extracellular polyhydroxybutyrate (PHB) depolymerases (PhaZs) — produced by a variety of microorganisms found [1-3] — remains an important and evolving research area. Their enzymatic activity results in the degradation of PHB, a natural biodegradable polymer with the potential to replace some of the currently widely used petroleum-based plastics [4], which increasingly accumulate in the environment [5].

Recombinant protein production is a powerful tool that allows the production of higher levels of proteins in expression systems such as *E. coli*-based ones compared to wild-type organisms; moreover, production optimization can be reached in *E. coli* through cell engineering, for example to redirect cell metabolic efforts towards product formation preferentially over growth without affecting transcriptional or translational efficiency [6], and even to achieve post-translational modifications like with the insertion of the *Campylobacter jejuni* glycosylation pathway into *E. coli* [7,8]. Also, other efforts in the expression platforms have allowed improved solubility, purification, and controlled expression, such as promoters design and the use of fusion tags [6]. These optimized recombinant technologies facilitate purification, the study of proteins in isolation, the conception of a platform to modify and improve them, and the development of new applications. In the case of PhaZs, such applications include biosensors — such as PhaZ-

based dye-loaded PHB time-temperature indicator [9] and pathogen detection-based platforms [10] — or recycling of biodegradable polymers, like improved whole-cell degradation through recombinant PhaZ (rPhaZ) co-expression with a protein that aids secretion [11].

The expression of rPhaZs has been performed over the years, with some examples including PhaZ2–PhaZ3 [12] and PhaZ7 [13] from *Pseudomonas lemoignei*, and PhaZ from *Caldimonas manganoxidans* [11,14]. In some cases, purification of heterologous PhaZs has also been performed: several PhaZs from *P. lemoignei* (PhaZ1–PhaZ5) [15,16], PhaZ7 and related mutants [17]), *Pseudomonas stuzeri* [18], *Alcaligenes faecalis* AE122 [19], *Marinobacter* sp. NK-1 [20], *Bacillus megaterium* N-18-25-9 [21], *Pseudomonas mendocina* DSWY0601 [22], and from *Cupriavidus* sp. (formerly denominated *Alcaligenes faecalis* T1 and *Ralstonia pickettii* T1 [23] — see APPENDIX A, section A.4) and related mutants [24-28]. However, these studies each required the development of specific methods for heterologous expression of specific PhaZs. In addition, in many cases affinity tags were not employed, requiring significant additional steps in the purification process [15,16,19,25-27]. These factors have limited the rapidity and scope of studies, even limiting the potential comparisons between PhaZs of diverse bacterial origins.

In this study, we established a platform for the rapid development of expression and purification processes for extracellular rPhaZs. This was demonstrated with five extracellular PhaZs from various bacterial strains from diverse environments, and with different enzyme properties. Predicted solubility and potential disulfide bond formation (necessary for many recombinant proteins to achieve proper conformation and retain activity [29]) of the rPhaZs produced were important criteria to select an *E. coli*-based

system, specifically the plasmid vector and expression strains, from the wide commercial offer of recombinant expression tools, as well as the screening induction conditions. The same platform and simple strategies — without the use specialized equipment — were employed for expression, purification, and preliminary comparison of degradation performance under different pH and temperatures.

4.3. Materials and methods

4.3.1. Bacterial strains and growth conditions

Cloning, expression, and PhaZ-producing bacterial strains — from the German Culture Collection (DSMZ) and the Japan Collection of Microorganisms (JCM)) — as well as their growth conditions including media (BD Becton Dickinson), and source can be found in Table 4.1a and b. Cell growth was monitored by measuring optical density of the cultures at 600 nm (OD_{600}) using a spectrophotometer (Biochrom, Ultrospec 50). Plating was performed on 1.5% w/v agar supplemented with the medium of interest.

Table 4.1. Bacterial strains and conditions.

Growth conditions and information of (a) PhaZ-producing strains and (b) cloning and expression strains.

a. PhaZ-producing strains information

Strain, PhaZ name (gene)	Sampling environment	Growth conditions	Source and Identification
Comamonas testosteroni 31A, PhaZ$_{Cte}$ (*phaZ$_{Cte}$*)	Soil from a greenhouse	Tryptic soy broth, 30 °C	DSMZ 6781
Cupriavidus sp., PhaZ$_{Csp}$ (*phaZ$_{Csp}$*)	Activated sludge obtained from the Toba sewage-treatment plant, Kyoto, Japan	Nutrient broth, 30 °C	JCM 10169
Marinobacter algicola DG893, PhaZ$_{Mal}$ (*phaZ$_{Mal}$*)	Laboratory culture of dinoflagellate *Gymnodinium catenatum* YC499B15	Marine broth, 28 °C	DSMZ 16394
Pseudomonas stutzeri, PhaZ$_{Pst}$ (*phaZ$_{Pst}$*)	Seawater, Jogashima, Kanagawa Pref., Japan	Nutrient broth, 30 °C	JCM 10168
Ralstonia sp., PhaZ$_{Rsp}$ (*phaZ$_{Rsp}$*)	Atmosphere in the laboratory, Japan	Nutrient broth, 30 °C	JCM 10171

b. Cloning and expression strains

Strain	Growth conditions	Source
NEB 5-alpha Competent *E. coli* (Subcloning Efficiency)	Luria–Bertani (LB) media supplemented with ampicillin or carbenicillin (100 µg/ml), 37 °C, 250 rpm	New England Biolabs (NEB)
E. coli ElectroMAX DH10B	LB media supplemented with ampicillin or carbenicillin (100 µg/ml), 37 °C, 250 rpm	ThermoFisher Scientific
E. coli Rosetta-gami B(DE3)	LB media in the presence of chloramphenicol (34 µg/ml), kanamycin (15 µg/ml), tetracyclin (12.5 µg/ml), and carbenicillin (50 µg/ml), 30 or 32 °C for precultures, 37 °C and 15 °C overnight during induction, 250 rpm. For plate cultures incubation was done for at least 24 h.	Novagen
T7 Express *lysY/I^q E. coli*	LB media supplemented with ampicillin or carbenicillin (100 µg/ml), 30 or 32 °C for precultures, 37 °C and 15 °C overnight during induction, 250 rpm	NEB

4.3.2. PhaZs constructs

Genomic DNA was extracted from the PhaZ-producing strains (RNA/DNA purification kit, Norgen Biotek for *C. testosteroni*, and GeneJET Genomic DNA Purification Kit, ThermoFisher Scientific for the other strains). Inserts were obtained by

amplifications of the mature *phaZ* genes (without signal peptides (SPs)) — with primers (5' to 3' direction) designed according to the sequences found in GenBank [30] (U16275.1 for $phaZ_{Cte}$, J04223.2 for $phaZ_{Csp}$, ABCP01000004.1 and EDM48791.1 for $phaZ_{Mal}$, AB012225.1 for $phaZ_{Pst}$, and D25315.1 for $phaZ_{Rsp}$), adding restriction sites *Eco*RI and *Xho*I (boldface in Table) through polymerase chain reaction (PCR) (T100 Thermal Cycler, Bio-Rad) and Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) (see Table 4.2). All PCR products were purified with QIAquick PCR Purification Kit (Qiagen).

Table 4.2. Primers used to produce mature rPhaZs.

phaZ gene	Primer number	5' to 3' sequence
$phaZ_{Cte}$	1	CAATTTACGACA**GAATTC**CGCCGTGCCGCTGGGGCAATACAACATT
	2	GCGATAAACAAT**CTCGAG**GGGGCAGGTACCGATCACGTAGTAGTTGCT
$phaZ_{Csp}$	3	CAATTTACCTCT**GAATTC**GGCCACGGCGGGGCCCGGTGCCT
	4	GCGATAAATGCT**CTCGAG**TGGACAATTGCCGACGATGTAGTAGCCGGCGGCCGTCT
$phaZ_{Mal}$	5	CTAACTTGCTAC**GAATTC**CGGCCAGACAGATTCCTACACCCTGCCACAG
	6	ATACAGCTAGCA**CTCGAG**GTTACACTGGCCGGCTTCGAAGATGCCGCT
$phaZ_{Pst}$	7	CAATTTACGACC**GAATTC**CGGGCAAACCTTCTCCTACACCTCCCCGCAA
	8	GTTATAAACAGT**CTCGAG**GTTGCTGCAGCGTCCGGCCTGGAAATG
$phaZ_{Rsp}$	9	CAATTTACCTC**GAATTC**AGCGGTCACCGCCGGGCCCGG
	10	GCGATAAATACA**CTCGAG**CGGGCAGTTGCCGATGACGTAGTAGCCGGC

Restriction digestions — using *Eco*RI and *Xho*I (NEB) — and ligations — using T4 DNA ligase (ThermoFisher Scientific) — were performed to obtain the PhaZs inserts and to incorporate them into the pET-22b(+) vector (Novagen). This plasmid includes an N-terminal peIB leader sequence for periplasm localization of the protein, and an optional C-terminal His-tag (included in the constructs); it is important to note that care should be taken not to include the SP sequence of native PhaZs. Transformation of *E. coli* was done by chemical transformation with heat shock at 42 °C for 30 s or electroporation with 0.1

cm gap cuvettes at 1.8 kV for 1 s (Gene Pulser, Bio-Rad), depending on the preparation of competent cells required. Both methods were followed by the addition of 250 µl of SOC medium (Super Optimal broth with Catabolite repression), incubation for 1 h at 37 °C and 250 rpm, and plating. The constructs were extracted with QIAprep Spin Miniprep Kit (Qiagen) and verified by DNA gel electrophoresis and sequencing (ABI 3730 DNA sequencer, Applied Biosystems). Constructs were inserted in *E. coli* Rosetta-gami B(DE3) cells, and in the case of PhaZ$_{Cte}$ and PhaZ$_{Mal}$ also in T7 Express *lysY/I^q E. coli*.

4.3.3. Induction screening and His-tag verification

Starter cultures from single colonies of the expression strain *E. coli* Rosetta-gami B(DE3), and when applicable T7 Express *lysY/I^q E. coli*, containing the constructs were grown in 5 ml LB with corresponding antibiotics (Table 4.1) until reaching an OD_{600} of approximately 0.5 (overnight incubation at or near 30 °C and 250 rpm is recommended). Small scale cultures (15 ml of LB with antibiotics) were inoculated with 1 ml of starter cultures and incubated at 37 °C until reaching an OD_{600} of approximately 0.6 (~5 h for *E. coli* Rosetta-gami B(DE3) and 2 h for T7 Express *lysY/I^q E. coli*). Cultures were separated into 3-ml aliquots, induced with isopropyl-β-d-thiogalactopyranoside (IPTG) at final concentrations ranging between 0.01 and 1 mM, and incubated overnight at 15 °C (for PhaZ$_{Cte}$ and PhaZ$_{Mal}$ incubations at 37 °C for 2 or 4 h were also tested). After incubation, 2 ml of induced cultures were centrifuged (10,000 × *g* and 4 °C for 10 minutes) and the pellets were kept at -20 °C. B-PER II Bacterial Protein Extraction Reagent (2X) (ThermoFisher Scientific), supplemented with lysozyme (1 mg/ml, Sigma-Aldrich) and DNAse I (5 units/ml, ThermoFisher Scientific) was used to obtain soluble fractions (150

µl/pellet); centrifugation was done at 21,130 × *g* and 4 °C for 30 minutes. The same procedure was performed with cells carrying the pET-22b(+) vector without the insert (negative controls) and samples before induction.

Characterization of the resulting soluble and insoluble fractions was then performed by treating samples, along with a Broad-Range protein standard ladder (6.5–210 kD) (Bio-Rad), for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, based on the methods described by Laemmli [31]. 2X Laemmli sample buffer (Bio-Rad) was added to each sample before they were boiled at 100 °C for 10 minutes. Loading volumes in the gel were normalized according to the OD_{600} of the cultures reached when they were stopped (before and at the end of the induction period). Samples were run in 12% polyacrylamide gels (Bio-Rad) for 40 minutes at constant voltage (200 V). The gels were washed three times with Milli-Q water for 10 minutes each and stained with PageBlue protein staining solution (ThermoFisher Scientific) for 1 h under gentle agitation. Finally, the gels were washed with Milli-Q water. Images of the gels were acquired under UV exposure (AlphaImager EC, Alpha Innotech) or with a regular camera. In the case of $PhaZ_{Cte}$ and $Phaz_{Mal}$, the presence of the His-tag was verified through Western blot analysis by using mouse anti-His6 monoclonal antibody, and goat anti-mouse DyLight 488 secondary antibody (Life Sciences).

4.3.4. Expression and purification of rPhaZs

30 mL of pre-cultures at OD_{600} of 0.5 were used to inoculate transformed *E. coli* Rosetta-gami B(DE3) in 1 L LB with antibiotics. Cultures were grown at 37 °C for approximately 5 h, until an OD_{600} of approximately 0.6 was reached. IPTG was added (at

0.05 and 1 mM for PhaZ$_{Mal}$, and 1 mM for all other PhaZs), and the cultures were incubated overnight at 15 °C. Once expression was ended, cultures were centrifuged at 10,000 × *g* and 4 °C for 10 minutes, and the pellets were stored at -20 °C. Protein extraction was performed on thawed pellets using 5 ml of the B-PER II mixture with Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X) (ThermoFisher Scientific) to obtain soluble fractions containing PhaZs.

Purification at 4 °C was performed using His GraviTrap columns (GE Healthcare). Equilibration was done with 10 ml of B-PER II. Extracted soluble fractions were applied to the column, followed by a wash with 10 ml of binding buffer (50 mM sodium phosphate, 500 mM NaCl, pH 7.4). All these three solutions contained imidazole at 20 mM (50 mM was also tested for PhaZ$_{Cte}$, PhaZ$_{Mal}$, and PhaZ$_{Rsp}$). His-tagged rPhaZs were eluted with 3 ml of elution buffer into 1-ml fractions (20 mM sodium phosphate, 500 mM NaCl, pH 7.4, with 150 mM imidazole for PhaZ$_{Mal}$ and 500 mM for all other PhaZs). Aliquots with 50% glycerol were made and stored at -20 °C. Purified rPhaZs were verified through SDS-PAGE and quantified with Bradford Protein Assay (microassay procedure, Bio-Rad) using bovine serum albumin as standard. Amicon Ultra 0.5 mL filters (Millipore) were used for PhaZ$_{Csp}$, PhaZ$_{Mal}$, and PhaZ$_{Rsp}$, which required further purification.

Buffer exchange was done with Amicon Ultra 0.5 mL filters or dialysis (Slide-A-Lyzer MINI Dialysis Devices, 20K MWCO, Thermo Scientific) was performed prior to assays in which imidazole and glycerol caused interference.

4.3.5. PHB plates rPhaZs activity comparison

Rapid PHB degradation assays during induction screening were performed by dispensing 100 µl of soluble fractions in cylindrical wells made in double-layer mineral medium/agar plates containing PHB (DSMZ list of recommended media for microorganisms, medium 474: 20 ml first layer, 10 ml second layer — described in Chapter 3, section 3.3.4). The plates were then incubated at 30 °C in a temperature-controlled incubator (Isotemp 500 Series, Fisher Scientific). PHB degradation was assessed by the presence of halos.

More precise analyses with purified rPhaZs were performed by varying pH and temperature. Since the double-layered PHB plates had pH 7.00, plates with pH 4.27 and 5.35 were obtained by replacing mineral medium with sodium acetate – acetic acid buffer solutions at the desired pH (both solutions at 0.2 M, see Millipore-Sigma Buffer Reference Center as reviewed from [32]). The agar and low pH buffer were autoclaved separately. The plates were pierced to produce cylindrical wells for the deposition of samples. Each rPhaZ was diluted to a concentration of ~2 µg/ml, and 100 µl were deposited for each condition in duplicates. Plates were incubated at 15 °C and 37 °C for pH 7.00, and at 37 °C for pH 4.27 and 5.35. Pictures were taken over four weeks of incubation. Again, degradation was assessed by the formation of transparent halos on the PHB plates and measuring the diameters using ImageJ 1.46r (National Institutes of Health, USA) (well diameters were subtracted).

4.4. Results and discussion

4.4.1. Selection of expression platform

An *E. coli*-based recombinant protein production system was selected, based on its relative success to produce rPhaZs [12,14-16,18-22] and the wide commercial offer of vectors and hosts. As a rapid way to take into consideration characteristics of the PhaZs to be produced, bioinformatics tools like predicted solubility and presence of disulfide bonds based on their amino acid sequence were analyzed in order to select a plasmid vector, expression hosts, and induction conditions. The sequences of the mature PhaZs were processed with a solubility predictor (PROSO II, [33,34]), and the theoretical isoelectric points (pI) and molecular weights (Mw) were calculated using the Compute pI/Mw tool from the ExPASy Bioformatics Resources Portal (SIB Swiss Institute of Bioinformatics) [35-37] (Table 4.3). The definition of the mature peptide sequence was done in agreement with the references of the experimentally studied PhaZs, and for PhaZ$_{Mal}$ (predicted PhaZ [2]) the analysis is described in Chapter 5, section 5.3.3. Since PhaZ$_{Cte}$ was classified as insoluble (predicted solubility score 0.503), and the predicted solubility scores for the other PhaZs (0.657–0.765) were relatively close to the 0.6 default PROSO II threshold for solubility, insolubility was considered as a potential drawback for recombinant production of PhaZs. In addition, extracellular PhaZs are known to be sensitive to dithiothreitol (DTT), which suggests that they contain cysteine residues that are likely to form disulfide bonds [38]; in fact the DiANNA 1.1 web server tool predicted several disulfide bonds for the mature PhaZs sequences in this study [39-41]. To overcome potential insolubility issues and the formation of inclusion bodies, induction was performed overnight at 15 °C. The

plasmid pET-22b(+) — previously used for fusion proteins with the substrate binding domain of PhaZ$_{Pst}$ [42], PhaZs from *P. mendocina* DSWY0601 [22], and *Bacillus* sp. NRRL B-14911 (and related variants) [43] — that contains the N-terminal peIB leader sequence for periplasm localization (a more favourable environment within the expression host for disulfide bond formation) of the heterologous protein was selected. In addition, we selected primarily the *E. coli* expression strain Rosetta-gami B(DE3) and as an alternative T7 Express *lysY/I^q* (which can potentially allow cloning and expression of toxic genes due to tight control of expression by *lacI^q* and control of T7 RNA Polymerase by lysozyme), both BL21 derivates designed to aid in expression of proteins that contain disulfide bonds and suitable for T7 expression (promoter of pET-22b(+)). Furthermore, Rosetta-gami B(DE3) *E. coli* contains the plasmid pRARE that supplies it with tRNAs for five rare codons (three present in PhaZ$_{Cte}$, one in PhaZ$_{C\text{sp}}$, and two in PhaZ$_{Mal}$, PhaZ$_{Pst}$, and PhaZ$_{R\text{sp}}$).

Table 4.3. PhaZs data.

Properties, sequence analyses [33-37,39-41,44], and qualitative expression and activity. Relative activity was tested on PHB plates where rPhaZs were deposited and displayed degradation as transparent halos; PHB plates containing rPhaZs were incubated for one week at 1. pH 7.00, 37 °C, 2. pH 7.00, 15 °C, and 3. pH 5.4, 37 °C.

Strain	PhaZ	PhaZ GenBank, reference	Theoretical mature PhaZ pI / Mw [kDa]	Predicted solubility score, class	Predicted disulfide bonds	% GC	Soluble expression level	Relative activity with PHB plates under different incubation assay conditions
Comamonas testosteroni 31A	PhaZ$_{Cte}$	U16275.1, [45]	7.64 / 50.6	0.503 insoluble	5	61	++	1. pH 7.0, 37 °C: + 2. pH 7.0, 15 °C: - 3. pH 5.4, 37 °C: ±-
Cupriavidus sp.	PhaZ$_{Csp}$	J04223.2, [25]	6.03 / 46.9	0.735 soluble	3	66	--	1. pH 7.0, 37 °C: + 2. pH 7.0, 15 °C: ±- 3. pH 5.4, 37 °C: ±
Marinobacter algicola DG893	PhaZ$_{Mal}$	ABCP01000004.1 and EDM48791.1, [46-48]	4.33 / 58.7	0.765 soluble	6	58	-	1. pH 7.0, 37 °C: + 2. pH 7.0, 15 °C: ± 3. pH 5.4, 37°C: --
Pseudomonas stutzeri	PhaZ$_{Pst}$	AB012225.1, [18]	5.32 / 57.5	0.657 soluble	6	66	+	1. pH 7.0, 37 °C: + 2. pH 7.0, 15 °C: - 3. pH 5.4, 37 °C: ---
Ralstonia sp.	PhaZ$_{Rsp}$	D25315.1, [49]	6.03 / 47.6	0.758 soluble	3	63	-	1. pH 7.0, 37 °C: + 2. pH 7.0, 15 °C: - 3. pH 5.4, 37 °C: ±

PhaZ expression levels on SDS-PAGE and activity on PHB plates: Very high (++), high (+), medium (±), low (-), very low (--), inactive (---).

Successful pET-22b(+)-PhaZs constructs were obtained for all cloned PhaZs. Sequencing of the mature *phaZ* gene inserts revealed that the sequences of PhaZ$_{Mal}$ and PhaZ$_{Pst}$ were the same as in the Genbank registers (Table 4.3), while some changes were found for PhaZ$_{Cte}$, PhaZ$_{Csp}$, and PhaZ$_{Rsp}$, that resulted in 3, 1, and 7 amino acid changes. These changes might be due to variations in the taxonomic strains [50] since their deposition.

4.4.2. Expression and purification of rPhaZs

Induction conditions for the successful constructs were screened mainly with the expression strain *E. coli* Rosetta-gami B(DE3). Expression was verified through SDS-

PAGE of the soluble and insoluble fractions (SF and IF) normalized according to OD_{600} at the point when induction was halted (data not shown). SDS-PAGE showed $PhaZ_{Cte}$ and $PhaZ_{Pst}$ were present in both SF and IF for inductions with 1 mM IPTG at 15 °C. Decreasing the IPTG concentration reduced insoluble rPhaZs, but the maximum SF expression was observed at 1 mM. $PhaZ_{Csp}$ expression was limited in both SF and IF, while $PhaZ_{Rsp}$ showed higher accumulation in the IF; the same phenomenon was observed for $PhaZ_{Mal}$. When inductions were carried at 37 °C, $PhaZ_{Cte}$ and $PhaZ_{Mal}$ were only present in the IFs.

PHB degradation was verified for SFs in PHB plates incubated at 30 °C — example shown in Figure 4.1 (i)) for $PhaZ_{Mal}$, for which clear zones were only observed for soluble fractions from cultures induced at 15 °C at 0.05 mM IPTG for T7 Express *lysY/I^q E. coli*, and at 0.4 or 1 mM IPTG for *E. coli* Rosetta-gami B(DE3)). T7 Express *lysY/I^q E. coli* was tested with $PhaZ_{Mal}$ to see if low expression could be due to gene toxicity, but expression was not improved and active enzyme could not be produced with induction above 0.05 mM IPTG even at 15 °C. A ring effect was observed likely due to high rPhaZ concentration caused by self-inhibition closer to the well, observed in previous investigations with PHB film [23,51,52]. The combined SDS-PAGE/PHB plates screening strategy is important because rPhaZs with low expression levels were not always observed by SDS-PAGE — the PHB plate assay helps confirm the presence of active rPhaZs in the SFs. For the other rPhaZs, also, only SFs from cultures induced at 15 °C displayed degradation activity. The best induction conditions were determined to be incubation at 15 °C with 1 mM IPTG for $PhaZ_{Cte}$, $PhaZ_{Csp}$, $PhaZ_{Rsp}$, and $PhaZ_{Pst}$ and 0.05 mM IPTG for $PhaZ_{Mal}$ (similar expression levels were obtained with 1 mM IPTG, but with lower purity). Removing the native SP

from the PhaZ sequence is a key step in diminishing the formation of inclusion bodies and avoiding completely insoluble PhaZs when using pET-22b(+) or plasmids that add signal sequences. As an example, Figure 4.1 (ii) shows the SDS-PAGE for an induction of T7 Express *lysY/I^q E. coli* with a construct containing PhaZ$_{Cte}$ with SP; cultures were induced with 0.1 mM IPTG and incubation was done at 15 °C overnight. Protein production is clearly visible but only observed in the induced IF; no significant amounts of PhaZ were observed in the SF, even under low temperature and low IPTG concentration conditions. This suggests the formation of inclusion bodies, likely due to confusion within the cell transporting mechanisms as two SPs were present. The proper use of pET-22b(+), which contains the peIB leader sequence for periplasm localization, might help achieve localization for proper protein folding.

The presence of the C-terminal His-tag was verified through Western Blot for SF and IF of PhaZ$_{Cte}$ and PhaZ$_{Mal}$ before proceeding to large-scale inductions for purification. Results from the SF and IF of PhaZ$_{Cte}$ induced at 15 °C overnight with 1 mM IPTG are presented in Figure 4.1 (iii) as example. Knowing that both of these PhaZ constructs successfully carried the His-tag, 1-L inductions were performed using the appropriate induction conditions. All rPhaZs could be separated through simple His-tag based purification (as confirmed by SDS-PAGE in Figure 4.1 (iv), (v)). Relative expression levels in the SF of each PhaZ can be observed in Figure 4.1 (v). These were qualitatively classified as very high for PhaZ$_{Cte}$, high for PhaZ$_{Pst}$, low for PhaZ$_{Mal}$ and PhaZ$_{Rsp}$, and very low for PhaZ$_{Csp}$ (Table 4.3). Purification, which was especially challenging for PhaZ$_{Mal}$, could be further improved using a combination of strategies including doing the equilibration, sample application, and wash steps with solutions containing 50 mM imidazole instead of

20 mM (which was tested with PhaZ$_{Cte}$, PhaZ$_{Mal}$, and PhaZ$_{Rsp}$, at the expense of losing some PhaZ), including an additional elution step with 150 mM imidazole instead of 500 mM for PhaZ$_{Mal}$, and using size exclusion columns (tested with PhaZ$_{Csp}$, PhaZ$_{Cte}$, PhaZ$_{Mal}$, and PhaZ$_{Rsp}$).

Attempts to improve yield of low expression constructs were made by inducing at higher IPTG concentrations and addition of ethanol in the induction stage [53]. None of these strategies led to improvements in expression levels. Rosetta-gami B(DE3) *E. coli* containing the PhaZ$_{Csp}$ construct had lower growth than other PhaZ constructs, which could suggest that *phaZ*$_{Csp}$ might negatively impact growth of this strain, including possible toxicity. However, differences in expression and solubility are difficult to attribute to particular features of these PhaZs. In the case of PhaZ$_{Mal}$ and PhaZ$_{Rsp}$ it could be related to their folding pattern, perhaps in these cases it is more difficult to reach native conformation in this expression system [8].

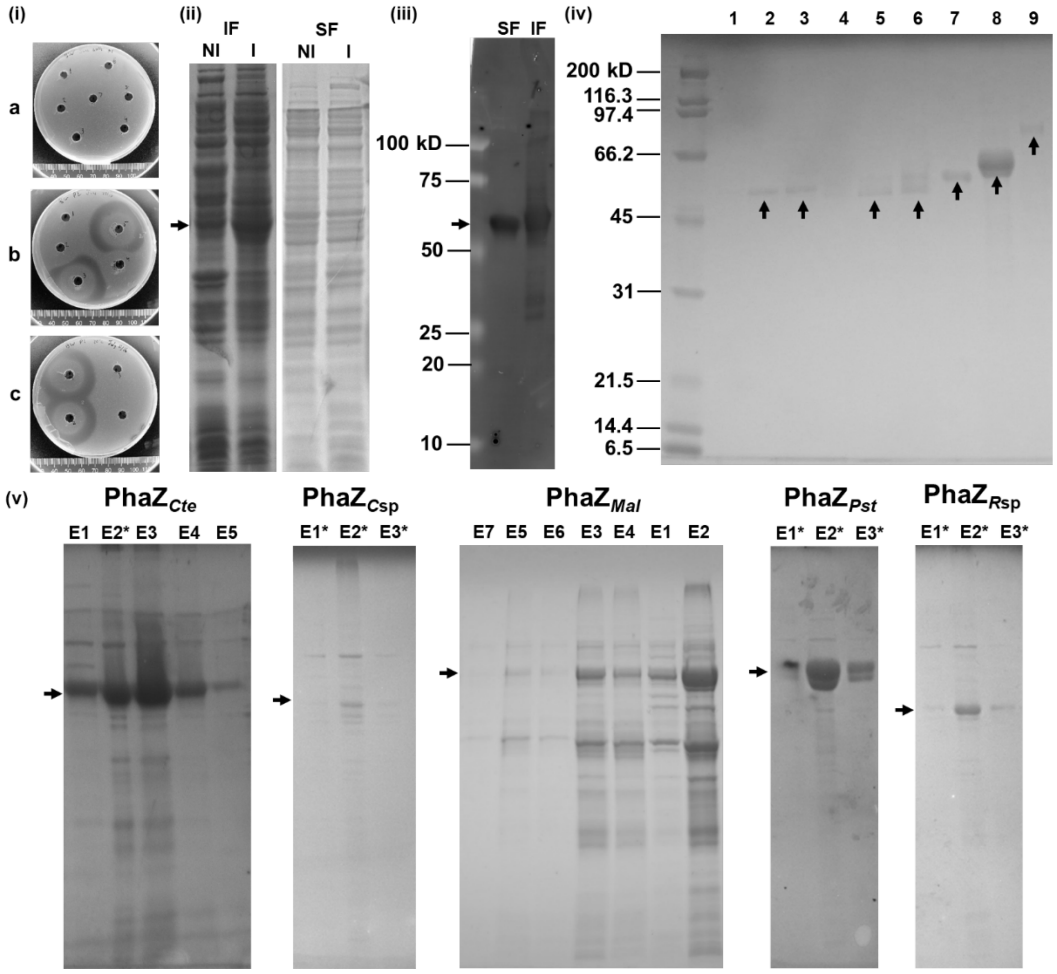

Figure 4.1. Examples of assays for production of rPhaZs.

(i) Induction screening of PhaZ$_{Mal}$ on PHB plates (SFs, 30 °C, 7 days incubation): (a) and (b) T7 Express *lysY/I^q E. coli* (induced with IPTG 0.05–1 mM, at 37 °C for 2 h and 15 °C overnight in duplicates), and (c) *E. coli* Rosetta-gami B(DE3) (induced with IPTG 0.01–1 mM, at 15 °C overnight). (ii) T7 Express *lysY/I^q E. coli* with construct containing PhaZ$_{Cte}$ with signal peptide, induction with 0.1 mM IPTG, at 15 °C overnight (NI: no IPTG added, I: IPTG added; 2 µl loaded for IF and 14 µl for SF). (iii) Western blot of SF and IF of PhaZ$_{Cte}$ containing a His tag (induced with 1 mM IPTG, at 15 °C overnight). (iv) Purified rPhaZs: lanes 1 to 9 are respectively: PhaZ$_{Csp}$ F, PhaZ$_{Csp}$ C, PhaZ$_{Csp}$, PhaZ$_{Rsp}$ F, PhaZ$_{Rsp}$ C, PhaZ$_{Rsp}$, PhaZ$_{Cte}$, PhaZ$_{Pst}$, PhaZ$_{Mal}$ C; where F: filtrate and C: concentrate from size exclusion columns. (v) SDS-PAGE of rPhaZs after imidazole elution (E: consecutive elution, asterisk indicates a 10-fold reduction factor in the loading volume of the elution fraction). Equilibration, sample application, and wash contained 20 mM imidazole, while the elution buffer contained 500 mM imidazole, except for PhaZ$_{Mal}$, for which 150 mM were used for E1 and E2, 100 mM for E3 and E4, ,200 mM for E5 and E6, and 500 mM for E7. Arrows point to rPhaZ locations.

4.4.3. Comparison of rPhaZs activity

A rapid methodology based on the use of PHB plates was used to compare rPhaZ activity. These plates can be fabricated at different pHs (additional buffers could be adapted) and incubated at multiple temperatures to assess the activity under various conditions, as can be observed in Figure 4.2. This method can also be used to provide a semi-quantitative assessment of activity based on the diameter of degradation halos formed. At 37 °C, all rPhaZs displayed similar activity, showing degradation from the first day of incubation (after two weeks halo diameters could no longer be measured), while longer incubation periods were required at 15 °C. PhaZ$_{Mal}$ presented an apparent higher activity in the PHB plates at 15 °C (halo observed after 1 day) compared to the other rPhaZs (halo observed after 6 days), which could be explained by its marine origin [48]. All enzymes were rendered inactive at pH 4.3 — no halos discernable — but PhaZ$_{Csp}$ and PhaZ$_{Rsp}$ retained significant activity at pH 5.4 compared to the other rPhaZs. This is consistent with their broad pH working ranges (PhaZ$_{Csp}$ is stable when stored at pH 5.0–8.0 [54] with optimum activity at pH 7.5 [55], and PhaZ$_{Rsp}$ optimum pH range is 5.0–6.00 [56]). Although, Yamada et al. showed PhaZ$_{Rsp}$ could retain some activity at pH 3.0 by monitoring decreases in turbidity of suspensions of PHB granules (around 20% relative activity with respect to its maximum) [56] — a method that has its own sensitivity and shortfalls.

PHB plates have been mostly used to screen for PHB degrading bacteria [1], but activity from expressed PhaZs has also been estimated by clearing zone diameters from spot tests on glass slides covered by PHB-agar mix [12,16]; this test is somewhat limited to short-term incubations (due to agar drying), but it is advantageous for preliminary

assessment and when small volumes of sample are available. From the results observed in the present study (Figure 4.2 (ii)), at 37 °C and pH 7.0 a similar degradation rate for all rPhaZs could be observed; at 15 °C and pH 7.0 the rate was slightly faster for PhaZ$_{Mal}$ and similar for the rest of the rPhaZs; at 37 °C and pH 5.4 the rate was superior for PhaZ$_{Csp}$ and PhaZ$_{Rsp}$, mild for PhaZ$_{Cte}$, and null for PhaZ$_{Mal}$ and PhaZ$_{Pst}$. These observations could be followed for weeks, if longer term incubations are required, but the trends could also be observed after a few days. These methods represent a powerful tool for screening recombinant and engineered PhaZs. This was demonstrated by Hiraishi et al. with the use of LB plates containing PHB granules, IPTG, and antibiotics to evaluate clear zone activity of PhaZ mutants [27].

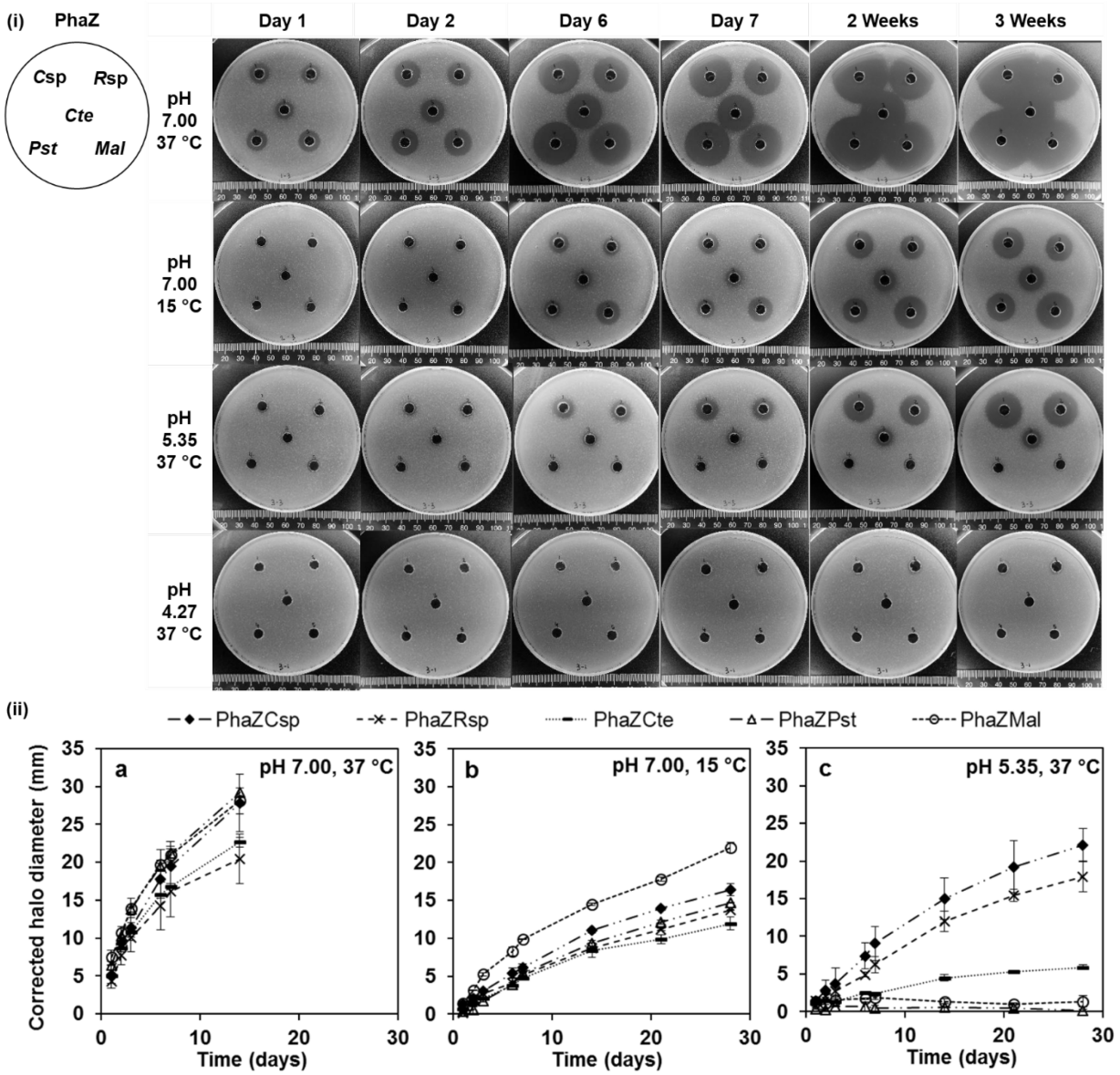

Figure 4.2. Degradation activity of rPhaZs on PHB plates.

rPhaZs concentration was ~2 µg/ml. Degradation as a function of time under different temperatures and pH values. (i) Halos indicate degradation.

(ii) Degradation halos diameter (corrected for well diameter). (a) pH 7.00, 37 °C; (b) pH 7.00, 15 °C; and (c) pH 5.35, 37 °C. No halos were observed at pH 4.27, 37 °C.

4.5. Conclusion

This study presents a streamlined platform for the rapid production of rPhaZs. Five active PHB-degrading extracellular PhaZs (PhaZ$_{Cte}$, PhaZ$_{Pst}$, PhaZ$_{Csp}$ PhaZ$_{Rsp}$, and PhaZ$_{Mal}$), from bacteria from diverse environments, were successfully produced in the soluble fraction of Rosetta-gami B(DE3) *E. coli*. An important aspect of the method requires the removal of the native SP sequence of PhaZ to avoid the production of inclusion bodies in the cells and inactive enzymes. Expression levels and purity varied for each enzyme — PhaZ$_{Cte}$ and PhaZ$_{Pst}$ saw highest expression — but all enzymes could be recovered, retained activity and were amenable to characterization. In addition, degradation activity could be easily assessed using PHB plates under a wide range of pH and temperatures. These incubations can be done in parallel as an initial basis for selecting PhaZs for diverse applications, such as scheduled PHB degradation or biosensors that act under specific temperature or pH conditions, or even to screen activity improvement through protein engineering. Both proposed methods, recombinant production and modified PHB plates assay, demonstrated versatility to produce and rapidly test diverse rPhaZs, which is typically reported for single rPhaZs or related variants, and showed potential to be employed with other PhaZs reported in the literature or novel ones to be discovered or synthesized.

4.6. Acknowledgements

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5. A Novel Polyhydroxybutyrate Depolymerase from the Marine Bacterium *Marinobacter algicola* DG893[‡]

5.1. Abstract

A putative extracellular polyhydroxybutyrate (PHB) depolymerase from the marine bacterium *Marinobacter algicola* DG893 ($PhaZ_{Mal}$) was produced heterologously in *Escherichia coli* and demonstrated to degrade PHB. Evaluated with common buffers (0.1 M potassium phosphate, Tris-HCl, and acetate buffer), $PhaZ_{Mal}$ showed highest activity degrading PHB film at low concentrations (0.5–1 μg/ml), neutral pH, and temperatures between room temperature and 37 °C, although it was still able to hydrolyze PHB at 15 °C. When tested under a wider array of buffers (at 0.5 μg/ml, 37°C, 24 h), $PhaZ_{Mal}$ activity increased by over 200% in mineral medium, and by 350% in mineral medium with added NaCl (4% w/v), compared to 0.1 M potassium phosphate. An analysis of the buffer components revealed that $CaCl_2$ and NaCl — both present in seawater — were responsible for these increases in activity, suggesting they likely act as activity mediators, which is consistent with other cases of PhaZs produced by marine microorganisms. On the other hand, mineral medium with added NaCl (4% w/v) reduced the activity of PhaZ from *Comamonas testosteroni* 31A ($PhaZ_{Cte}$) — a well characterized soil strain — by approximately 70%. Degradation was compared at similar PHB film mass loss levels between $PhaZ_{Mal}$ and $PhaZ_{Cte}$. The results revealed similar morphological and small

[‡] A version of this chapter will be submitted for publication as: Diana I Martínez-Tobón, Brennan Waters, Anastasia L Elias, and Dominic Sauvageau. A novel polyhydroxybutyrate depolymerase from the marine bacterium *Marinobacter algicola* DG893.

changes in PHB molecular weight at the highest degradation level analyzed, and that although the main degradation product was 3HB monomer for both enzymes, a larger proportion of dimers and even trimers were observed with PhaZ$_{Mal}$, suggesting a difference in mechanism.

Keywords: Polyhydroxybutyrate (PHB) degradation, PHB depolymerase (PhaZ), *Marinobacter algicola* DG893, enzyme ion activation.

5.2. Introduction

Polyhydroxyalkanoates (PHAs) — of which poly(3-hydroxybutyrate) (PHB) is the most abundant and widely studied — are biodegradable, biocompatible, and thermoplastic polyesters. They are biosynthesized and accumulated by several bacteria as carbon and energy storage molecules [1-3]. As their properties are similar to those of polypropylene and polyethylene [1], these biopolymers have the potential to replace many petroleum-derived plastics in applications such as packaging and coatings [4]. Many microorganisms biodegrade PHB as a substrate for carbon and energy. They do so through the action of PHB depolymerases (PhaZs) which break the polyester down to monomers and oligomers to make its utilization possible [1,2].

In addition to successful commercial ventures — e.g. Biomer and Goodfellow [5-8] — significant research and development efforts are ongoing to help further improve the commercial viability of PHB production. This includes, for instance, modelling optimized fermentation strategies [9], and producing PHB with methanotrophic bacteria using methane and methanol as feed, instead of sugars [10,11]. Metabolic engineering approaches have also been attempted, by using PHB-producing microorganisms for co-

production of PHB with other value-added products such as rhamnolipids [12] or L-tryptophan [13] (as reviewed by Kumar and Kim [14]). In addition, the use of Clustered regularly interspaced short palindromic repeats interference (CRISPRi) technology in the producer *Halomonas* sp. TD01 has enhanced PHB synthesis through specific gene repression [15]. Considering our consumption trend and waste management practices — e.g. in 2010 the amount of plastic waste released to the marine environment was estimated to be between 4.8–12.7 metric tons per year [16,17] — as the use of PHB-based products becomes more widespread, its presence in the marine environment is bound to increase. In this sense, investigating the ability of marine strains to degrade PHB, and the enzymes involved in this process, are important topics of interest.

Extracellular PhaZs (from here onwards PhaZ refers to extracellular PHB depolymerases in this chapter, unless otherwise specified) from saltwater bacteria have been identified, and in some cases characterized. These comprise PhaZs from *Comamonas testosteroni* YM1004 [18], *Pseudomonas stutzeri* YM1006 [19,20], *Alcaligenes faecalis* AE122 [21,22], *Streptomyces* sp. SNG9 [23], *Marinobacter* sp. NK1 [24,25], *Nocardiopsis aegyptia* [26], and *Shewanella* sp. [27]. Since the number of characterized PhaZs from saltwater bacteria is limited, amplifying this portfolio is a worthwhile task, especially considering that the search for PhaZs with diverse properties will allow better understanding of the fate of PHB in specific environments — such as low temperature or high salt content — the development of PHB recycling or disposal processes, and the conception of PhaZ-based technologies.

In a previous study (Chapter 3) [28], we performed an initial exploration of the capacity of various marine strains with predicted PhaZ activity according to the PHA

Depolymerase Engineering Database (PHA-DED) [29] (*Alteromonas macleodii*, *Loktanella vestfoldensis, Marinobacter algicola* DG893, and *Oceanibulbus indolifex* Hel45) to degrade PHB films as sole carbon source in liquid cultures. Of all these strains, only *M. algicola* DG893 was able to cause PHB films mass loss (up to 11% after two weeks of exposure) under the conditions tested (Figure 3.2b and c) — 10 ml mineral medium (German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ), Brunner, medium 457) cultures with 2% w/v PHB film (cut into 1 cm^2 pieces) and 10% v/v inoculum from cultures grown in marine broth (sections 3.3.3 and 3.3.4) — [28]. *M. algicola* DG893 is a marine, Gram-negative, moderately halophilic, and hydrocarbon-degrading bacterial strain isolated from the dinoflagellate *Gymnodinium catenatum* YC499B15 [30].

In this study, we explore a broader range of culture conditions with the four selected marine strains, by testing modified versions of mineral medium and marine broth to supply the strains with compounds that might be necessary for growth — given their marine origin — and that are not present in regular mineral medium. Moreover, the potential of *M. algicola* and its extracellular PhaZ enzyme to degrade PHB was evaluated through recombinant protein production. The predicted *phaZ* gene from *M. algicola* was cloned in *Escherichia coli*, expressed, and the enzyme ($PhaZ_{Mal}$) was purified, and characterized under a wide range of conditions and buffers typically used with PhaZs (0.1 M potassium phosphate, Tris-HCl, and acetate buffer) to determine their effect on PHB film degradation by $PhaZ_{Mal}$. Activity was also assessed by analyzing the composition of regular and modified mineral medium. In addition, comparisons were made between $PhaZ_{Mal}$ and the well-characterized PhaZ from the soil strain *Comamonas testosteroni* 31A ($PhaZ_{Cte}$),

including morphology of PHB film after enzyme erosion, weight-average molecular weight (Mw) and polydispersity index (Mw/Mn) through Gel Permeation Chromatography (GPC), analysis of degradation products through high-performance liquid chromatography (HPLC) and reverse phase HPLC-mass spectrometry (RP-HPLC- MS) — to study the proportion of degradation products yielded using each enzyme — and the effect of 0.1 M potassium phosphate and modified mineral medium on the activity of each enzyme.

5.3. Materials and methods

5.3.1. PHB film fabrication

PHB film was produced through solvent casting of PHB pellets (BRS Bulk Bio-pellets, Bulk Reef Supply, Golden Valley, USA) dissolved in glacial acetic acid (Fisher Scientific) and processed at 140 °C, as described in Anbukarasu et al. [31].

5.3.2. Bacterial strains and growth conditions

Bacterial strains, medium (BD Becton Dickinson) and growth conditions, used in this study can be found in Table 5.1. Cell growth was monitored by optical density at 600 nm (OD_{600}) measured using a spectrophotometer (Biochrom, Ultrospec 50). Plating was performed on 1.5% w/v agar supplemented with the medium of interest. For details of *E. coli* strains for production of recombinant $PhaZ_{Mal}$ (NEB 5-alpha Competent *E. coli* (Subcloning Efficiency), and *E. coli* Rosetta-gami B(DE3)) see Chapter 4, Table 4.1.

For the marine strains, starter cultures were initiated from single colonies taken from marine broth agar plates placed in 20 ml of marine broth at the indicated growth conditions for 48 h.

Table 5.1. Bacterial marine strains and growth conditions used in this study.

Strain	Growth conditions	Reference or source
Alteromonas macleodii collection number: 20772	Marine broth, 25°C, 150 rpm	Japan Collection of Microorganisms (JCM)
Loktanella vestfoldensis collection number: 21637	Marine broth, 25°C, 150 rpm	JCM
Marinobacter algicola DG893 collection number: 16394	Marine broth, 28°C, 150 rpm	DSMZ
Oceanibulbus indolifex Hel45 collection number: 14862	Marine broth, 28°C, 150 rpm	DSMZ

The marine strains with predicted PhaZ activity were tested with PHB film as carbon source in modified mineral medium and modified marine broth. The original compositions of mineral medium and marine broth, provided by DSMZ and BD Becton Dickinson, are listed on Table 5.2. The modifications consisted of adding 4% w/v NaCl to the original mineral medium composition, and of removing the carbon sources (peptone and yeast extract) from the marine broth. For the modified marine broth, $C_6H_5FeO_7$ was replaced with 0.1082 g of $(NH_4)_5\ Fe(C_6H_4O_7)_2$.

Table 5.2. Original mineral medium and marine broth compositions.

Mineral medium[a]		Marine broth[b]	
Component	**Amount**	**Component**	**Amount**
Na_2HPO_4	2.44 g	Peptone*	5.0 g
KH_2PO_4	1.52 g	Yeast Extract*	1.0 g
$(NH_4)_2SO_4$	0.50 g	$C_6H_5FeO_7$†	0.1 g
$MgSO_4•7H_2O$	0.20 g	NaCl	19.45 g
$CaCl_2•2H_2O$	0.05 g	$MgCl_2$	5.9 g
Trace element solution SL-4	10 ml	$MgSO_4$	3.24 g
Distilled water	1000 ml	$CaCl_2$	1.8 g
Trace element solution SL-4:		KCl	0.55 g
EDTA	0.50 g	$NaHCO_3$	0.16 g
$FeSO_4•7H_2O$	0.20 g	KBr	0.08 g
Trace element solution SL-6	100 ml	$SrCl_2$	0.034 g
Distilled water	900 ml	H_3BO_3	0.022 g
Trace element solution SL-6:		Na_2O_3Si	0.004 g
$ZnSO_4•7H_2O$	0.10 g	NaF	0.0024 g
$MnCl_2•4H_2O$	0.03 g	$(NH_4)(NO_3)$	0.0016 g
H_3BO_3	0.30 g	Na_2HPO_4	0.008 g
$CoCl_2•6H_2O$	0.20 g	Distilled water	1000 ml
$CuCl_2•2H_2O$	0.01 g		
$NiCl_2•6H_2O$	0.02 g		
$Na_2MoO_4•2H_2O$	0.03 g		
Distilled water	1000 ml		

[a] 4% w/v NaCl was added to produce modified mineral medium.
[b] For the modified marine broth: * indicates components that were removed, and † the component that was replaced by 0.1082 g of $(NH_4)_5 Fe(C_6H_4O_7)_2$.

Inocula consisting of 1 ml of pre-cultures were added to 9 ml of mineral medium with 4% w/v NaCl or modified marine broth, containing close to 0.1 g of PHB films cut into approximately 1 cm^2 pieces (pre-weighed using an analytical balance (Denver Instruments, SI-234)). Cultures were incubated at 30 °C and 150 rpm for 14 days (Ecotron, Infors). PHB film degradation was evaluated by % mass loss when the cultures were terminated. All experiments were performed in triplicate.

5.3.3. Expression, purification, and quantification of recombinant PhaZ$_{Mal}$

Genomic DNA (gDNA) was extracted (GeneJET Genomic DNA Purification Kit, ThermoFisher Scientific) from a fully-grown pre-culture of *M. algicola*. In order to build a construct based on the PhaZ prediction for *M. algicola*, we used the sequence submitted by Green et al. [30,32,33] (GenBank [34] ABCP01000004.1 locus tag MDG893_02490, protein sequence GenBank EDM48791.1, annotated by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline Group), listed in the PHA-DED [29] under the Homologous family e-dPHAscl (type 1), homologous family 9. After doing a signal peptide (SP) prediction analysis for Gram-negative bacteria with SignalP 4.1 Server (default settings) [35,36] (predicted cleavage site between amino acids 32 and 33 (AFA-GQ D=0.900 D-cutoff=0.570 Networks=SignalP-noTM)), forward and reverse primers — 5'-CTAACTTGCTAC**GAATTC**CGGCCAGACAGATTCCTACACCCTGCCACAG-3' and 5'-ATACAGCTAGCA**CTCGAG**GTTACACTGGCCGGCTTCGAAGATGCCGCT -3' carrying *Eco*RI and *Xho*I restriction sites (boldface), respectively — were designed and used in gene amplification through polymerase chain reaction (PCR) (T100 Thermal Cycler, Bio-Rad) with Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific). Restriction digestions and ligation were performed to obtain the insert and to clone the construct by incorporation into the pET-22b(+) expression vector (Novagen).

The nucleotide sequence for mature PhaZ$_{Mal}$ — confirmed by sequencing (ABI 3730 DNA sequencer, Applied Biosystems) with T7 promoter primer (5'-TAATACGACTCACTATAGGG -3') and middle primers (5'-GCAGCTGATAACACCCGGGA-3', 5'- ACCGAGACCTGCGAGCAGGC-3', and 5'-ACTGCTGGGGCTTCTGGTTT-3') — can be found in Figure 5.1, along with the

prediction from SignalP 4.1 Server. In addition, mature PhaZ$_{Mal}$ domains were identified through a Multiple Alignment using Fast Fourier Transform (MAFFT) [37] of the protein sequences from PhaZ from *Marinobacter* sp. NK-1 (PhaZ$_{Msp}$, Genbank BAC15574.1 [24]) and PhaZ from *Pseudomonas stutzeri* (PhaZ$_{Pst}$, Genbank BAA32541.1 [19]) — 75 and 66% identical to PhaZ$_{Mal}$ deducted amino acid sequence — with Geneious version 11.1.4 [38], also used to generate the graphical representation.

The sequenced nucleotides are in agreement with the direct submission by Green et al. under GenBank ABCP01000004.1 locus tag MDG893_02490, and protein sequence GenBank EDM48791.1 [30,32,33].

Figure 5.1. PhaZ$_{Mal}$ nucleotide and protein sequences.

See next page.

Amino acids are written below the second nucleotide of the corresponding codon. The sequence for the SP predicted by SignalP 4.1 Server [35,36] was taken from GenBank ABCP01000004.1 locus tag MDG893_02490, and protein sequence GenBank EDM48791.1 (Green et al., direct submission [30,32,33]). Annotated domains (catalytic domain (Cat), linker domain (link), substrate binding domain (SBD)) were determined based on the protein sequences of PhaZ$_{Msp}$ Genbank (BAC15574.1 [24]) and PhaZ$_{Pst}$ (Genbank BAA32541.1 [19]).

For a detailed description of cloning, expression, and purification of PhaZ$_{Mal}$, see sections 4.3.1–4.3.4. Induction was performed at 0.05 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubation was done overnight at 15 °C. During purification, the concentration of imidazole added to the solutions was 50 mM for equilibration, sample application, and wash steps, and 150 mM for the elution step. Buffer exchange of the eluted fraction to 0.1 M potassium phosphate pH 7.4 was done with Amicon Ultra 0.5-mL filters (molecular weight cut off 50K (Millipore)). Purified PhaZ$_{Mal}$ was verified through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis — based on the methods described by Laemmli [39] — to verify protein size and relative purity, and concentration was quantified with Bradford Protein Assay (microassay procedure, Bio-Rad) using bovine serum albumin as standard. The purified protein was aliquoted and stored at -20 °C for further characterization. At different stages of PhaZ$_{Mal}$ production, degradation was qualitatively tested using double-layer mineral medium agar plates supplemented with PHB powder (DSMZ list of recommended media for microorganisms, medium 474: 20 ml first layer, 10 ml second layer; described in Chapter 3, section 3.3.4), pierced to produce cylindrical wells for the deposition of 100 µl per sample tested. Activity was qualitatively assessed by observation of PHB degradation halos.

5.3.4. PhaZ$_{Mal}$ Activity

PHB films (1.3 cm × 1.3 cm) were weighed (Mettler Toledo, XS 105 Dual range) and autoclaved before being exposed to 1 ml of PhaZ$_{Mal}$ in 0.1 M potassium phosphate pH 7.4 at concentrations ranging from 0 to 10 µg/ml for 24 h at 37 °C without agitation. The same experimental procedure was used for PhaZ$_{Mal}$ at a concentration of 0.5 µg/ml at temperatures ranging from 4 to 60 °C, and with different buffers (0.1 M acetate buffer, 0.1 M potassium phosphate, and 0.1 M Tris-HCl) at pH ranging from 3.1 to 9.9, for 24 h.

The impact of medium composition on activity was explored by using the following buffers: a) 0.1 M potassium phosphate, b) 0.1 M sodium phosphate, c) regular mineral medium, d) mineral medium with added NaCl (4% w/v), e) mineral medium with Na_2HPO_4 replaced by K_2HPO_4, f) mineral medium with Na_2HPO_4 replaced by K_2HPO_4 and without $CaCl_2 \bullet 2H_2O$, g) mineral medium with Na_2HPO_4 replaced by K_2HPO_4 and without $CaCl_2 \bullet 2H_2O$ and trace solution SL4, h) mineral medium without $CaCl_2 \bullet 2H_2O$, and i) mineral medium without $CaCl_2 \bullet 2H_2O$ and with added NaCl (4% w/v) — for components of mineral medium see Table 5.2. The conditions for film exposure were 0.5 µg/ml PhaZ$_{Mal}$ at 37 °C for 24 h.

PhaZ$_{Mal}$ at 0.5 µg/ml diluted separately in 0.1 M potassium phosphate and 0.1 M sodium phosphate (each at pH 7.2), was stored for 24 and 48 h at room temperature (RT), 30, and 37 °C. The enzymatic solutions were then incubated for 24 h with PHB film at 37 °C. Mass loss and activity were assessed after incubation using the method described above.

For all experiments, after incubation, the buffers were removed and the PHB films were washed two times with sterile Milli-Q water, dried at 50 °C for at least 48 h, and weighed again. Control experiments were conducted without enzymes. PhaZ$_{Mal}$ activity was reported as a % mass loss per time (evaluated at 24 h) and experiments were performed in triplicates (unless otherwise stated).

5.3.5. Analysis of partially degraded PHB film

PHB films were exposed to 0.5 µg/ml PhaZ$_{Mal}$ or PhaZ$_{Cte}$ in mineral medium with added 4% w/v NaCl at 37 °C. Degradation was allowed to proceed until samples lost approximately 0.3, 2, and 13% of their initial mass. Negative controls with buffer alone incubated for 30 and 48 h were also included. The partially degraded films were washed and dried before being weighed and analyzed by scanning electron microscopy (SEM) (Zeiss Sigma 300 VP-FESEM, beam voltage 2.0 kV, SE2 detector). These films were also analyzed by gel permeation chromatography (GPC, Water Breeze) to determine Mw and Mw/Mn. HPLC-grade chloroform was used as the mobile phase (0.3 ml/min flow rate), and the GPC was equipped with 3 Waters Styragel® HR columns (HR1 with a molecular weight measurement range of 10^2 – 5 × 10^3 g/mol, HR2 with a molecular weight measurement range of 5 ×10^2 – 2 × 10^4 g/mol and HR4 with a molecular weight measurement range of 5 × 10^3 – 6 × 10^5 g/mol) and a guard column was used. The columns were heated to 40 °C during the analysis. Mw was determined by calibration with linear narrow molecular weight distribution polystyrene standards (PSS Polymer Standards Service GmbH, molecular weights ranging from 682 g/mol to 2,520,000 g/mol) and the GPC was equipped with a differential refractive index (RI 2414) detector.

5.3.6. PHB film degradation products

After degradation, the liquid fraction containing the degradation products from PHB films was kept at -20 °C for further analysis. The degradation products were analyzed by HPLC using the method developed by Gebauer and Jendrossek [40], based on the fatty acid detection method from Durst et al. [41], in which 3HB oligomers were derivatized into bromophenacyl derivatives for analysis. Briefly, frozen samples containing PHB film degradation products were thawed and acidified with HCl prior to extraction with ethyl acetate (1:1, 3 times, left to settle for at least 1 h). The organic phase containing the soluble degradation products was evaporated and 100 µl of 0.1 M trimethylamine in acetone were added. The samples were vortexed and evaporated under nitrogen gas. Derivatization was performed by the addition of 150 µl of 10 mM 2,4′-dibromoacetophenone (bromophenacyl bromide, BPB) in acetonitrile, and 160 µl of 1.9 mM 18-crown-6 in acetonitrile which catalyzes the reaction. The samples were heated to 80 °C for 90 min in a test tube with a sealed lid, vortexed several times, and cooled down to RT. In order to have enough volume for analysis, 600 µl of acetonitrile was added to all samples. The detection of PHB hydrolysis products was done with an Agilent LC 1200 HPLC system equipped with a UV detector at 254 nm (Agilent) and an Eclipse XDB-C18 4.6 mm ID × 250 mm (5 µm) 80Å column (Agilent), following the detection method described by Gebauer and Jendrossek [40]. Calibration was performed using 3HB standard dilutions (5–100 mM).

In addition, RP-HPLC- MS was performed. For this technique, the method was adapted for an ultra performance liquid chromatography (UPLC) small column, using an Agilent 1200 SL HPLC System with a Kinetex C18 reverse-phase analytical column (2.1x50 mm, 1.7 µm particle size, 100 Å) (Phenomenex), thermostated at 50 °C, followed

by UV detection at 254nm and mass spectrometric detection. An aliquot of 2 µL was loaded onto the column at a flow rate of 0.5 ml/min and an initial buffer composition of 70% mobile phase A (10 mM ammonium formate, pH 4 in 98% water and 2% methanol) and 30% mobile phase B (GC resolv methanol). Analytes were eluted using a linear gradient from 30% to 100% mobile phase B over a period of 12 minutes, held at 100% mobile phase B for 2 minutes to remove all analytes from the column and 100% to 30% mobile phase B over a period of 1 minute. Mass spectra were acquired in positive mode of ionization using an Agilent 6220 Accurate-Mass TOF HPLC/MS system equipped with a dual sprayer electrospray ionization source with the second sprayer providing a reference mass solution. Mass spectrometric conditions were drying gas 10 L/min at 325 °C, nebulizer 25 psi, mass range 100–2000 Da, acquisition rate of ~1.03 spectra/sec, fragmentor 150V, skimmer 65V, capillary 3800V, instrument state 4GHz High Resolution. Mass correction was performed for every individual spectrum using peaks at m/z 121.0508 and 921.0098 from the reference solution (buffer alone: mineral medium with 4% NaCl). Data acquisition was performed using the Mass Hunter software package (version B.04.00). Analysis of the HPLC-UV-MS data was done using the Agilent Mass Hunter Qualitative Analysis software (version B.07.00).

5.4. Results

5.4.1. Cloning, expression, and purification of recombinant PhaZ*Mal*

Of all the marine strains tested — *A. macleodii*, *L. vestfoldensis, M. algicola* DG893, and *O. indolifex* Hel45 — only *M. algicola* led to significant PHB film mass loss in both modified media: reaching 11% in modified marine broth, and 62% in mineral medium with added NaCl (Figure 5.2a). Based on these results, PhaZ*Mal* was selected for further investigation of its activity and degradation mechanism.

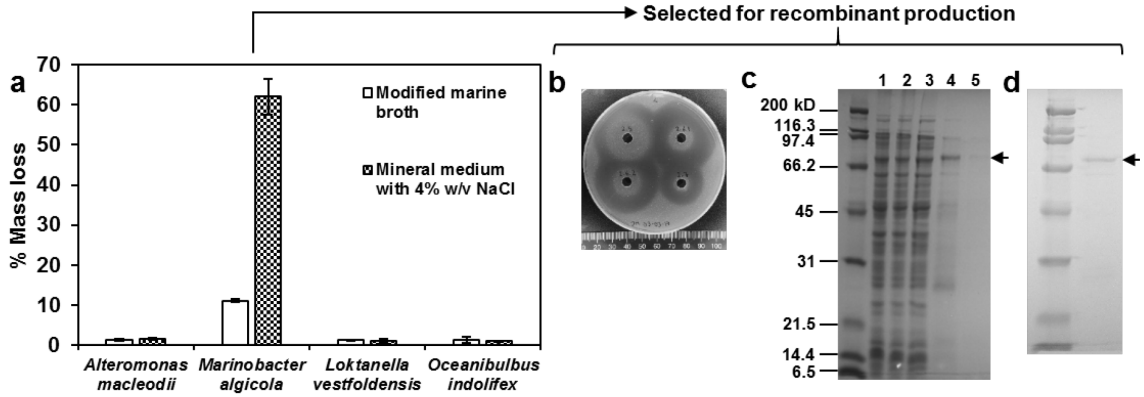

Figure 5.2. PhaZ*Mal* expression and purification after recombinant production.
(a) PHB film % mass loss from marine strain cultures with PHB as sole carbon source in modified marine broth and mineral medium with 4% w/v NaCl; cultures were incubated at 30 °C and 150 rpm for 14 days. (b) PHB plate showing degradation from PhaZ*Mal* purification fractions (wash and elutions with 150 and 500 mM imidazole). (c) PhaZ*Mal* SDS-PAGE purification fractions 1: soluble fraction (pre-purification), 2: unbound fraction, 3: wash fraction, 4: elution fraction with 150 mM imidazole, 5: elution fraction with 500 mM imidazole. (d) Purified PhaZ*Mal* after size exclusion column step. Arrows point approximate location of PhaZ*Mal*.

The pET-22b(+) construct carrying the predicted *phaZ*$_{Mal}$ gene sequence (with its SP removed) was successfully built and expressed in *E. coli* Rosetta-gami B(DE3). PhaZ$_{Mal}$ recovered after purification displayed hydrolysis on PHB plates incubated at 37 °C for 7 days (Figure 5.2b). The different fractions of the purification process (Figure 5.2c), and the resulting eluted fraction (150 mM imidazole) after buffer exchange with 50K filters (Figure 5.2d) were analyzed through SDS-PAGE, which showed that the filtration step aided reducing the presence of contaminating bands. Approximately 200 µg of PhaZ$_{Mal}$ were obtained per liter of induced culture using the process described.

5.4.2. Activity assessment

PHB film % mass loss per h was used to assess PhaZ$_{Mal}$ activity under a wide range of conditions (Figure 5.3). When investigating the activity of degradation as a function of enzyme concentration at 37 °C in 0.1 M potassium phosphate, pH 7.4, the greatest activity (approximately 0.08% mass loss per h) was reached between 0.5 to 1 µg/ml (Figure 5.3a). It should also be noted that beyond this region, activity decreased down to 0.02% mass loss per h at an enzyme concentration of 10 µg/ml.

The effect of pH on activity was investigated for pH ranging from 3.14 to pH 9.92 using three different buffers (acetate buffer, potassium phosphate, and Tris-HCl, all at 0.1 M) and 0.5 µg/ml enzyme at 37 °C for 24h (Figure 5.3b). First, it is important to note that there was good agreement for results obtained with different buffers at overlapping pH. The highest activity was observed at near neutral pH (pH 7.4, 2.5% mass loss per h). Lower and higher pH resulted in decreases in activity — reduced to less than 0.03% mass loss per h below pH 5 and above pH 8. Negative controls (buffers without enzyme) were evaluated

with acetate buffer pH 3.14, potassium phosphate pH 5.97 and 7.97, and with Tris-HCl pH 9.92; all had values below 0.02 % mass loss per h.

The third parameter investigated was temperature (Figure 5.3c), which ranged from 4 to 60 °C in 0.1 M potassium phosphate with 0.5 µg/ml PhaZ$_{Mal}$, pH 7.4, for 24 h. The optimal temperature under the conditions tested was found to be 30 °C with approximately 0.15% mass loss per h evaluated at 24 and 48 h. No activity was observed at 4, 50, and 60 °C during the incubation period. Negative controls were incubated at 4, 30, and 60 °C for 24 h and the maximum PHB % mass loss per h obtained was 0.02.

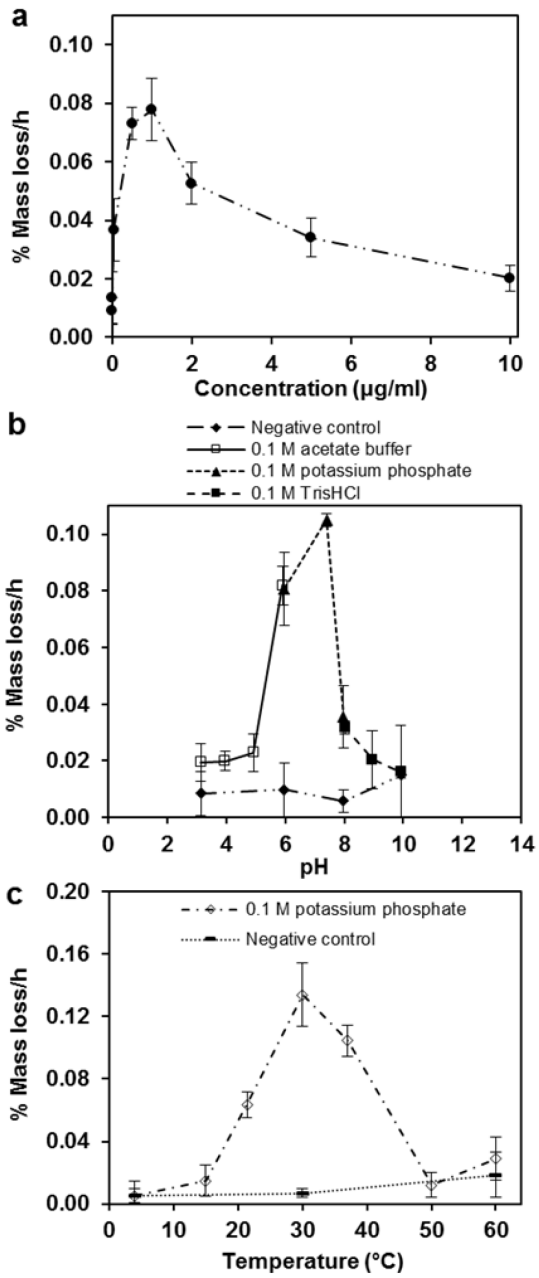

Figure 5.3. PHB film % mass loss per h as an effect of PhaZ*Mal* concentration, pH and temperature.

Incubations took place for 24 h with variations of: (a) enzyme concentration (PhaZ*Mal* concentrations 0–10 µg/ml in 0.1 M potassium phosphate, pH 7.4, incubated at 37 °C); (b) pH (evaluated for pH 3.14–9.92; 0.1 M acetate buffer: pH 3.14- 5.92; 0.1 M potassium phosphate: pH 5.97-7.97; 0.1 M Tris-HCl: pH 8.02-9.92. All with 0.5 µg/ml PhaZ*Mal*, at 37 °C; negative controls were evaluated with acetate buffer pH 3.14, potassium phosphate pH 5.97 and 7.97, and with Tris-HCl pH 9.92); and (c) temperature (evaluated for 4–60 °C with 0.5 µg/ml PhaZ*Mal*, in 0.1 M potassium phosphate, pH 7.4); negative controls were incubated at 4, 30, and 60 °C. All experiments were performed in triplicates.

A more in-depth survey of medium composition was conducted with 0.5 µg/ml PhaZ$_{Mal}$ incubated at 37 °C for 24 h (Figure 5.4a). In a) 0.1 M potassium phosphate pH 7.4, 0.10 ± 0.01% mass loss per h was measured. Activity was found to be greater in five media. In b) 0.1 M sodium phosphate pH 7.2, degradation values reached around 0.22 ± 0.05% mass loss per h. In c) mineral medium pH 7.06, it reached 0.21 ± 0.01% mass loss per h, while d) mineral medium with added 4% w/v NaCl pH 6.52 led to the greatest degradation at 0.37 ± 0.04% mass loss per h. In addition, when Na_2HPO_4 was replaced by K_2HPO_4 in mineral medium (e)), PHB film mass loss was reduced to 0.12 ± 0.02% mass loss per h; for f), when $CaCl_2$ was removed and along with g) the trace element solution SL4 (Na_2HPO_4 replaced by K_2HPO_4), or for h) when $CaCl_2$ was removed from the regular mineral medium, degradation was only slightly greater than negative controls (maximum 0.037 ± 0.01% mass loss per h). However, in the case of i) mineral medium without $CaCl_2$ but with added NaCl (4% w/v), degradation did occur with 0.22 ± 0.04% mass loss per h. Negative controls (performed with potassium and sodium phosphates, mineral medium, mineral medium with 4% w/v NaCl, and mineral medium with Na_2HPO_4 replaced by K_2HPO_4) were below 0.02 ± 0.004% mass loss per h.

A preliminary evaluation of the stability of PhaZ$_{Mal}$ was performed with 0.1 M potassium phosphate and 0.1 M sodium phosphate, both at pH 7.2, with 0.5 µg/ml PhaZ$_{Mal}$ incubated at RT, 30, and 37 °C for 24 and 48 h. Activity was assessed by further incubating samples for 24 h with PHB film at 37 °C and comparing activity by normalizing with the fresh enzyme values (0.05 ± 0.005 and 0.17 ± 0.02% mass loss per h for potassium and sodium phosphate respectively) (Figure 5.4b). Storage in potassium phosphate led to higher variability in activity after storage, likely due to its low absolute activity. Activity

decreased to 60% of its initial value after the longest storage at RT and 30 °C. On the other hand, storage in sodium phosphate led to more abrupt decreases, to approximately 40% its initial value after 24 h at the same temperatures. Enzyme deactivation was complete for storage in either buffer at 37 °C, regardless of the storage time.

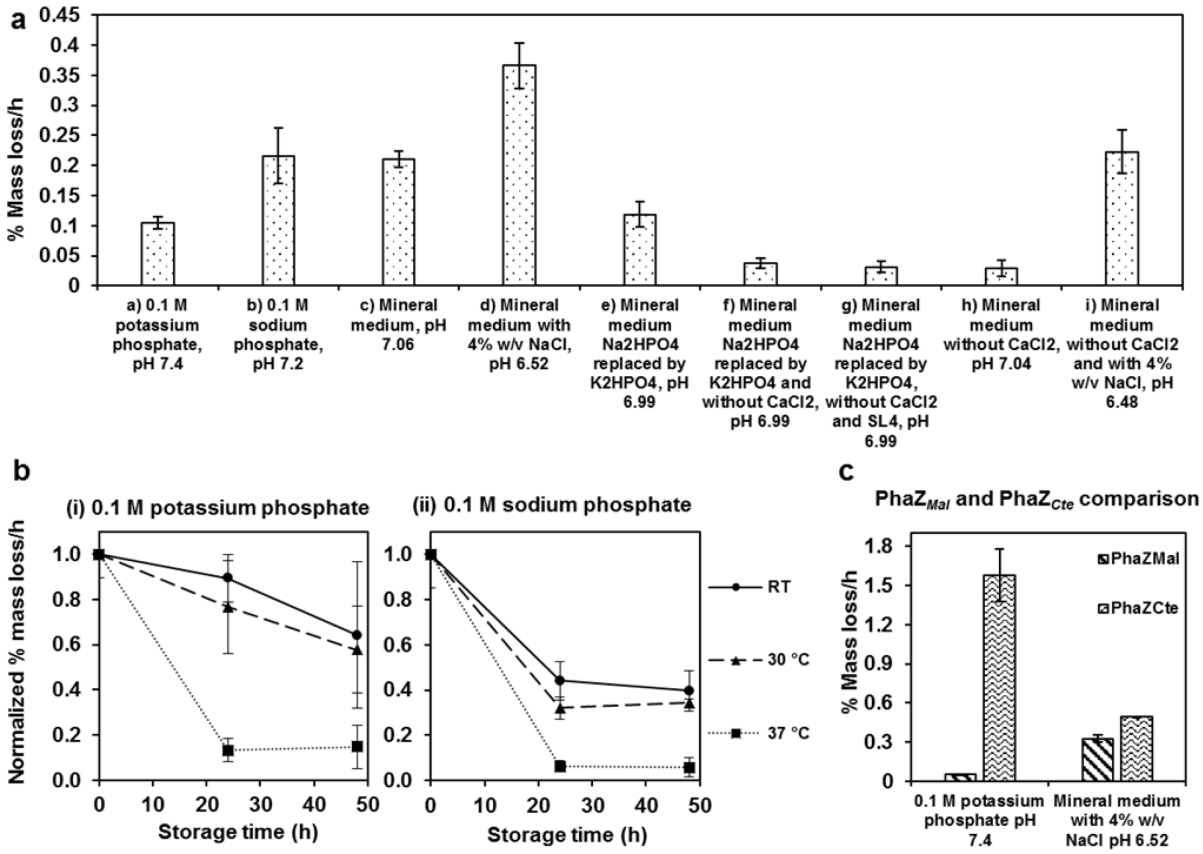

Figure 5.4. Influence of medium composition on PhaZ*Mal* activity and stability.
(a) PHB film degradation after 24 h of incubation with 0.5 µg/ml PhaZ*Mal* at 37 °C; negative controls (performed with potassium and sodium phosphates, mineral medium, mineral medium with 4% w/v NaCl, and mineral medium with Na_2HPO_4 replaced by K_2HPO_4) showed activity below 0.02 ± 0.004 % mass loss per h. (b) Normalized activity of 0.5 µg/ml PhaZ*Mal* after storage for 0, 24 and 48 h at RT, 30, and 37 °C in (i) 0.1 M potassium phosphate and (ii) 0.1 M sodium phosphate, both at pH 7.2. (c) Activity comparison between 0.5 µg/ml PhaZ*Mal* (diagonal bars) and PhaZ*Cte* (zigzag bars) — performed in duplicates — with PHB film incubated for 48 h at 37 °C in 0.1 M potassium phosphate pH 7.4 and mineral medium with 4% w/v NaCl; negative controls were below 0.02 ± 0.003 % mass loss per h. Data is presented as mean ± SD (n=3 for all except Figure 5.5c where n=2).

5.4.3. PHB film degradation by PhaZ$_{Mal}$ and PhaZ$_{Cte}$

The degradation activities of PhaZ$_{Mal}$ and PhaZ$_{Cte}$ were compared by incubating PHB films with the enzymes for 48 h at 37 °C in 0.1 M potassium phosphate pH 7.4 and mineral medium with 4% w/v NaCl (Figure 5.4c). In potassium phosphate, the activity of PhaZ$_{Cte}$ was approximately 30 times that of PhaZ$_{Mal}$, while in mineral medium supplemented with NaCl it was only 1.5 times greater. Mineral medium with 4% w/v NaCl had opposite effects on the two enzymes: PhaZ$_{Mal}$ activity increased by approximately 600% while PhaZ$_{Cte}$ activity decreased by 70%. Subsequent comparisons of degradation by the two enzymes were done by reaching similar levels of % mass loss.

SEM micrographs of dry PHB film, and PHB films exposed to buffer alone, buffer containing PhaZ$_{Mal}$ and buffer containing PhaZ$_{Cte}$ can be seen in Figure 5.5. As expected, the surface of the films appears to become rougher as the films are attacked by the enzymes. At the highest degradation level investigated (13.5% mass loss), PHB films showed similar morphology patterns regardless of the enzyme used; however, at the lower degradation levels (~0.5% and 2.2% mass losses), slight variations can be observed, especially at 20,000× magnification. For PhaZ$_{Cte}$ the morphology appears smoother than for PhaZ$_{Mal}$ for both low degradation levels. Higher porosity was observed for PhaZ$_{Mal}$ at 0.5% 20,000× magnification, but this is probably due to porosity heterogeneity from the film as can be observed for PhaZ$_{Mal}$ at 0.5% 5,000× and from Figure B.2 in APPENDIX B. At 2.2% the jagged phases (likely originating from the removal of amorphous but not crystalline regions of the spherulites) observed for both enzymes at 13.5% were already reached for PhaZ$_{Mal}$,

while it seems to have taken further degradation for PhaZ$_{Cte}$ for the pronounced whiskers to develop.

Qualitative analysis of derivatized degradation products by HPLC and RP-HPLC-MS was performed for identification — solvent extraction limits quantitative determination since 3HB is only partially recovered during the ethyl acetate extraction process [40]. Figure 5.6 shows results for UPLC results for a negative control, a 3HB standard, and the liquid fractions of partially degraded PHB films (~2 and ~12% mass loss) exposed to PhaZ$_{Mal}$ and PhaZ$_{Cte}$. Figure 5.6a shows the chromatogram obtained from the liquid fraction of PHB film incubated in mineral medium with 4% NaCl for 30 h at 37 °C (negative control) for reference, displaying a peak of unreacted BPB at 3.7 min (present in all other chromatograms) and peaks at 1.9 and 3.1 min that originate from the mineral medium with 4% NaCl (confirmed by UPLC of medium alone — data not shown). Figure 5.6b shows a chromatogram for a reference sample/calibration standard consisting of 50 mM 3HB (91.5% purity). Peaks are found for 3HB monomer and dimer — confirmed by detection in extracted ion chromatograms (EIC), and by the expected respective mass to charge ratios (m/z) of the quasimolecular ions ($[M + H]^+$, $[M + NH_4]^+$, and $[M + Na]^+$) in the MS spectra (peaks present in double form with increments of two due to the two bromine isotopes ^{79}Br and ^{81}Br). Additional peaks at 5.1, 5.9, 6.5, and 7.1 min are assumed to be higher oligomers based on masses determined by HPLC/MS. Approximate proportions of the degradation products were determined from the corresponding peak areas for UPLC chromatograms detected through UV (Figure 5.6), and from compound abundance determined by MS analysis (data not shown). For PHB films degraded to 2.4% mass loss after incubation with PhaZ$_{Mal}$ (Figure 5.6c), monomer and dimer were detected

through UV at an approximate proportion of 2:1, while MS analysis revealed the presence of 3HB monomer, dimer, and trimer in proportions of 9:29:1. For PHB film that reached 2.2% mass loss after incubation with PhaZ$_{Cte}$ (Figure 5.6d), monomer and dimer were also detected through UV at an approximate proportion 7:1, with MS analysis showing a 2:1 proportion of the same compounds. No significant amount of trimer was detected compared to the negative control. For PHB film that reached 12.1% mass loss after incubation with PhaZ$_{Mal}$ (Figure 5.6e), the monomer and dimer proportions through UV were 4:1, while MS indicated monomer, dimer, and trimer in proportions 12:2:1. And for PHB film that reached 10.9% mass loss after incubation with PhaZ$_{Cte}$ (Figure 5.6f), monomer and dimer showed a proportion of 3:1 through UV, and MS analysis showed monomer, dimer, and trimer with a 61:11:1 proportion. These results suggest that the end product for both enzymes is the 3HB monomer, but that PhaZ$_{Mal}$ leads to significant levels of dimer and trimer, especially in the early stages of degradation.

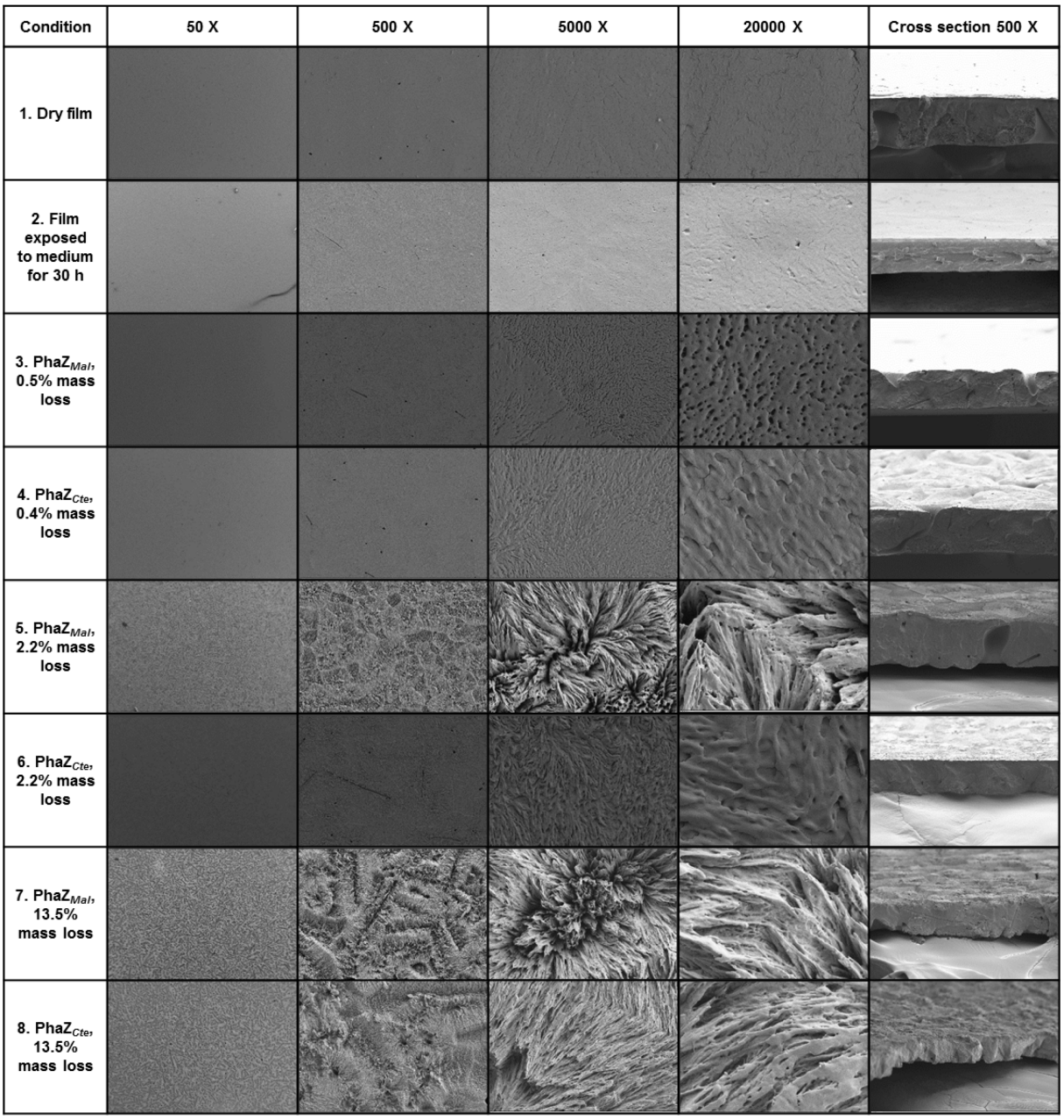

Figure 5.5. Scanning electron microscopy micrographs of PHB film exposed to PhaZ*Mal* and PhaZ*Cte* reaching different levels of degradation, as determined by mass loss.

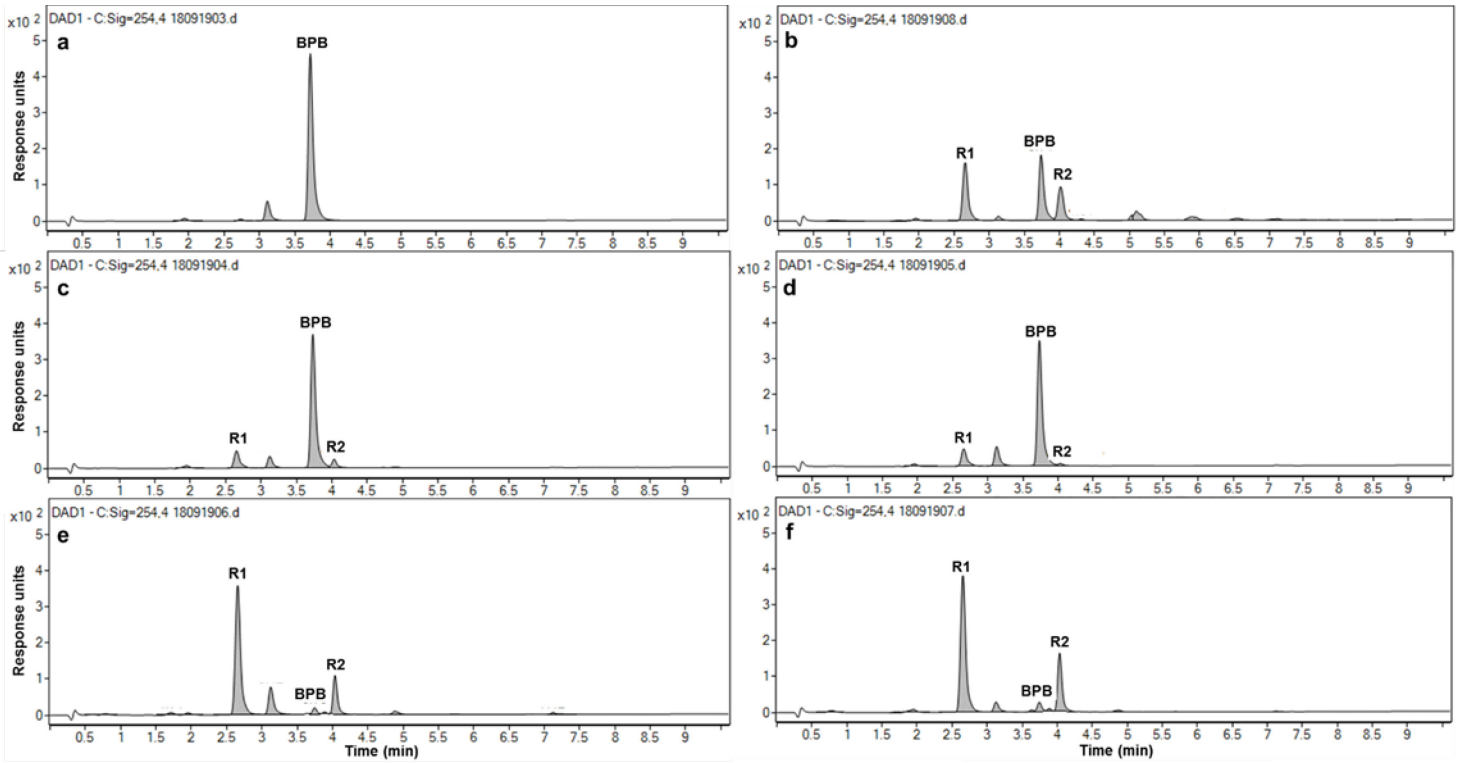

Figure 5.6. UPLC of BPB derivates detected at 254 nm.

(a) Negative control: Liquid fraction of PHB film incubated in mineral medium with 4% NaCl for 30 h at 37 °C. (b) Reference sample: 50 mM 3HB 91.5% purity, 3HB monomer (2.7 min) and dimer (4.0 min) (correlated with HPLC/MS data), denominated R1 and R2 respectively; additional peaks at 5.1, 5.9, 6.5, and 7.1 min are assumed to be higher oligomers based on masses determined by HPLC/MS. (c) and (d) Liquid fractions of PHB film incubated with PhaZ$_{Mal}$ and PhaZ$_{Cte}$, respectively, in mineral medium with 4% NaCl to a mass loss of ~2%. (e) and (f) Liquid fractions of PHB film incubated with PhaZ$_{Mal}$ and PhaZ$_{Cte}$, respectively, in mineral medium with 4% NaCl to a mass loss of ~12%. Peaks at 1.9 and 3.1 min originate from the mineral medium with 4% NaCl (confirmed by UPLC of medium alone — data not shown). Unreacted BPB generated a peak at 3.7 min.

GPC results (Table 5.3) showed that Mw and Mw/Mn of the partially-degraded films was almost unchanged from the unexposed films (in agreement with superimposed chromatograms, data not shown), with only very small apparent decreases in Mw for samples degraded by PhaZ$_{Mal}$ (~14% mass loss) and PhaZ$_{Cte}$ (~2 and 14% mass loss).

Table 5.3. GPC results (Mw and Mw/Mn) of PHB film exposed to PhaZ$_{Mal}$ and PhaZ$_{Cte}$ reaching different levels of degradation, as determined by mass loss.

GPC result	Treatment							
	1. Dry film	**2. Film exposed to medium for 30 h**	**3. PhaZ$_{Mal}$, 0.3% mass loss**	**4. PhaZ$_{Cte}$, 0.3% mass loss**	**5. PhaZ$_{Mal}$, 2.5% mass loss**	**6. PhaZ$_{Cte}$, 1.9% mass loss**	**7. PhaZ$_{Mal}$, 13.1% mass loss**	**8. PhaZ$_{Cte}$, 13.5% mass loss**
n	1	2	3	3	3	3	3	3
Mw [Da]	2.59×10^5	$2.37 \times 10^5 \pm 3 \times 10^3$	$2.5 \times 10^5 \pm 2 \times 10^4$	$2.42 \times 10^5 \pm 5 \times 10^3$	$2.3 \times 10^5 \pm 1 \times 10^4$	$2.03 \times 10^5 \pm 1 \times 10^4$	$1.9 \times 10^5 \pm 1 \times 10^4$	$2.1 \times 10^5 \pm 1 \times 10^4$
Mw/Mn	2.40	2.42 ± 0.03	2.52 ± 0.1	2.57 ± 0.03	2.5 ± 0.1	2.35 ± 0.09	2.33 ± 0.1	2.33 ± 0.03

5.5. Discussion

In this study, a group of marine bacterial strains carrying putative genes for extracellular PhaZs that were previously tested in low salt media (Chapter 3) [28] were further assessed in modified media with higher salinity. Again, even in these media, only *M. algicola* showed PhaZ activity; no significant PHB degradation was detectable for *A. macleodii*, *L. vestfoldensis*, and *O. indolifex* (Figure 5.2a). The predicted PhaZ$_{Mal}$ was thus cloned, expressed and its activity was characterized under several conditions. Given the high similarities of the mature peptide of PhaZ$_{Mal}$ with PhaZs from *Marinobacter* sp. (PhaZ$_{Msp}$) and *Pseudomonas stutzeri* (PhaZ$_{Pst}$) (75 and 66%, respectively), the enzyme domains of PhaZ$_{Mal}$ were identified (Figure 5.1), revealing that it possesses a cadherin-type

linker and is among the extracellular PhaZs group that contain two SBDs [19,21,24,42-44]. From the amino acid sequence, the expected size of the mature peptide was 58.7 kDa but SDS-PAGE analysis showed a size of ~70 kDa (Figure 5.2c and d) — similar to size of PhaZ$_{Msp}$ as obtained by SDS-PAGE [25]. Such discrepancies have been observed for other PhaZs [21,45].

We proceeded to characterize PhaZ$_{Mal}$ activity through mass loss measurements of PHB film exposed to the enzyme. The impact of pH was assessed using 0.1 M potassium phosphate pH 7.4 along with 0.1 M acetate buffer and 0.1 M Tris-HCl at different pH. The conditions of highest activity were found to be 0.5–1 µg/ml PhaZ$_{Mal}$, pH 7.4 (with reduced activity below pH 5 and above pH 8) and 30 °C (activity was reduced but retained at RT and 15 °C). The best pH and temperature values obtained are in accord with the optimal growth conditions for *M. algicola* [30], although enzymatic degradation was not examined in that study. These results are consistent with those obtained for the PhaZs with high sequence similarity: PhaZ$_{Msp}$ was reported to hydrolyze PHB film at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) [25], and activity with PHB granules was measured in 50 mM Tris–HCl buffer (pH 7.5) [24], while PhaZ$_{Pst}$ was shown to retain some activity at RT and 15 °C [19].

It also seemed apparent from preliminary stability tests (Figure 5.4b), that the storage of diluted PhaZ$_{Mal}$ (0.5 µg/ml) at RT and 30 °C led to reductions in activity, while storage at 37 °C led to inactivation. However, the enzymes could be stored for months at -20 °C at higher concentrations (around 50–100 µg/ml) without significant loss in activity. Activity, even at optimal conditions, was relatively low compared to some other PhaZ, for instance PhaZ$_{Cte}$, (Figure 5.4c), at least in 0.1 M potassium phosphate pH 7.4. As other

media were explored to establish the range of activity of the enzyme, Ca^{2+} and Na^{+} were found to be potential activity mediators (through the presence of $CaCl_2$ and NaCl). PhaZ$_{Mal}$ activity with PHB film increased by over 200% in mineral medium (which contains Ca^{2+}), and by 350% in mineral medium with added NaCl (4% w/v), compared to 0.1 M potassium phosphate (which has neither Ca^{2+} nor Na^{+}) after 24 h incubation at 37 °C.

An initial hypothesis for the positive effect of NaCl in PhaZ$_{Mal}$ activity could be that *M. algicola* needs Na^{+} for growth (observed between 1 and 9% w/v NaCl, optimum 3–6%) [30]. However, *Marinobacter* sp. also needs sodium for growth, but PhaZ$_{Msp}$ did not show changes in enzymatic activity in the presence of NaCl up to 0.5 M [25], which indicates that for PhaZ from a halophilic strain does not necessarily require Na^{+}. On the other hand, the activity of PhaZ$_{Msp}$ has been shown to increase by 132 and 172% in the presence of 1 mM Mg^{2+} and Ca^{2+}, respectively [25]. PhaZ from the marine bacterium *A. faecalis* AE122 — a strain that also needs Na^{+} for growth — also showed activation by the addition of seawater (highest at 30%, and unaltered from 50 to 100% seawater) or of NaCl, $MgCl_2$, and $CaCl_2$ at 200, 12, and 3.8 mM, respectively (50% of the molar concentrations found in seawater) [22]; similar levels of activity were also found when NaCl, Na_2SO_4, and KCl were at high concentrations (200 mM), while lower concentrations (10 mM) were needed for other salts like $SrCl_2$, $MgCl_2$, and $CaCl_2$ [22]. The authors concluded that, for this enzyme, PhaZ activity is greatly enhanced by the synergistic effect of salt combinations found in seawater [22]. In the present study, $CaCl_2$ was present at 0.26 mM in mineral medium, a concentration sufficient to have a noticeable positive effect on activity (Figure 5.4a); in contrast $MgSO_4$ was present at 0.4 mM, much lower than the concentration used by Kita et al. (1995). The strongest effect was observed when NaCl was

added to a concentration of 0.68 M in mineral medium, for which a synergistic effect could also be occurring between NaCl and $CaCl_2$.

A more extreme case has been reported for PhaZ from the marine strain *Shewanella* sp. JKCM-AJ6,1α [42]: the wild type enzyme lacks both SBDs, but high salt concentrations in the marine environment have been suggested to promote hydrophobic interactions between the enzyme and PHB, making it function as well as other PhaZs in seawater [42]. It is possible that the presence of the different salts in the studied media for $PhaZ_{Mal}$ could be aiding in non-specific interactions that make it easier for the enzyme to interact with PHB. Another explanation could be generated from the fact that halophilic enzymes require high salt concentrations for activity and generally have an excess of acidic amino acids over basic ones, with the hypothesis that a negative surface charge makes them more soluble and flexible under high salt concentrations [46,47]. On the other hand, non-halophilic enzymes tend to aggregate and become rigid at high salt concentration [46,47]. This could explain why $PhaZ_{Cte}$ (active at pH 4.8–10.6) underperformed in mineral medium with 4% w/v NaCl (pH 6.52), compared to 0.1 M potassium phosphate (pH 7.4) (Figure 5.4c). The isoelectric points predicted from the amino acid sequences of $PhaZ_{Mal}$ and $PhaZ_{Cte}$ are 4.33 and 7.64, respectively — calculated using the Compute pI/Mw tool from the ExPASy Bioformatics Resources Portal (SIB Swiss Institute of Bioinformatics) [48-50] — which indicates that $PhaZ_{Mal}$ is a more acidic protein. Mevarech et al. explained — through the analysis of the enzyme malate-dehydrogenase and the 2Fe–2S protein ferredoxin of a extremely halophilic archaeon — that such high surface charges are mainly neutralized by tightly bound water dipoles, while high salt concentrations are required to overcome weak binding of the salt to specific sites on the surface of folded enzymes, which

helps stabilize the active conformation [46]. In addition, Takaku et al. proposed that based on a dramatic enhancement in activity in the presence of 1 mM $CaCl_2$ (7-fold increase) [51] Ca^{2+} was as a cofactor for PhaZ from *Bacillus megaterium* N-18-25-9. They also found that 100 mM KCl inhibited enzyme activity [51]. Based on these factors and the results presented here, it is possible that Na^+ and Ca^{2+} could be cofactors of $PhaZ_{Mal}$.

$PhaZ_{Mal}$ and $PhaZ_{Cte}$ degradation resulted in progressive surface erosion with a whiskers pattern of PHB (Figure 5.5). These are consistent with the favourable degradation of the amorphous areas of the spherulites that comprise the film and subsequent attack of the crystalline zones, and with no apparent changes in the inner part of the films, similar to patterns described previously from SEM studies with PhaZs [52-54]. The lack of degradation in the inner parts of the films (Figure 5.5, cross sections) supports the very small decrease in Mw of the PHB films measured through GPC (Table 5.3). A previous study has also shown that surface erosion of PHB film degraded by 8.5% after exposure to seawater from a coral reef aquarium led to no decrease in Mw from superimposed GPC chromatograms [55]. Doi et al. also found that after enzymatic degradation with PhaZ from *Alcaligenes faecalis* T1 (currently *Cupriavidus* sp., see APPENDIX A, section A.4 [28]) in 0.1 M phosphate buffer at 37 °C for up to 20 h, PHB films had reduced mass but the Mw remained almost unchanged, concluding that PhaZ only hydrolyses the polymer chains on the surface contrary to simple hydrolysis where random inner chain scission occurs on the long term [53]. The caveat here is that once most of the polymer chains are hydrolyzed, the Mw will eventually decrease.

The analysis of degradation products released after incubation of PHB films with $PhaZ_{Mal}$ led to identify the 3HB monomer, dimer, and trimer as degradation products

through HPLC and HPLC/MS. Samples at low levels of degradation showed a high proportion of dimer, while once mass losses reached ~12% the main product was the monomer. When comparing with the products resulting from hydrolysis by PhaZ$_{Cte}$, which mostly produced monomer at all stages of degradation, the high proportion of dimer (and trimer) produced by PhaZ$_{Mal}$ suggests these enzymes undergo different hydrolytic mechanisms, which could help explain the differences in SEM morphology observed at early degradation (~2% mass loss) by PhaZ$_{Mal}$ and PhaZ$_{Cte}$ (Figure 5.5), as perhaps the removal of products larger than the monomer could aid in revealing more defined crystalline regions, which are eventually exposed for both enzymes as degradation progresses. For PhaZ$_{Cte}$, the main product is the monomer which is in agreement with previous findings through a 3HB dehydrogenase-dependent optical assay [56], although some dimer was also detected in a small proportion with the more sensitive HPLC and HPLC/MS performed in the present study. PhaZ$_{Mal}$ seems to favour hydrolysis of the second or third monomeric unit, which suggests it has endo- and exo-depolymerase degradation mechanism, as has been showed for other PhaZs [2,57]. This being said, PhaZ$_{Mal}$ eventually hydrolyses the polymer and oligomers to 3HB, which agrees with the degradation products of PHB film by PhaZ$_{Msp}$, found to be monomer (in the highest proportion), dimer, trimer during degradation for up to 4.5 h at 37 °C in 0.1M potassium phosphate buffer (pH 7.4) with 2 μg/ml enzyme [25]. In a study of PhaZ$_{Pst}$, end point enzymatic products of PHB film were monomer and dimer, with bigger proportion of monomer [20], and later in a kinetic analysis with water soluble 3HB oligomers (up to tetramer) it was shown that the catalytic site of PhaZ$_{Pst}$, recognizes at least two 3HB units as substrate and has preference to bind to three 3HB units [19].

5.6. Conclusion

From the work presented in this study, we experimentally showed that *M. algicola* DG893 produces an extracellular PhaZ — as suggested by a preliminary study (Chapter 3) [28] — validating the function of a protein that was previously only reported as a predicted PhaZ [29,32,33]. Comparison with $PhaZ_{Cte}$ (from soil origin) revealed that the mechanisms of both enzymes show differences when degrading PHB film, regarding morphology of the film, and primary hydrolysis products — during early degradation stage. In addition, buffer composition, in this case mineral medium with 4% w/v NaCl, boosted activity of $PhaZ_{Mal}$ while reducing that of $PhaZ_{Cte}$ likely due to their protein sequence and conformation. In general, $PhaZ_{Mal}$ showed slower activity levels compared to $PhaZ_{Cte}$, but there is wide potential for further activity improvement by exploration of broader concentrations of ions like Na^+, Mg^{2+}, and Ca^{2+}. Additional exploration of *M. algicola* DG893 and $PhaZ_{Mal}$ could lead to their utilization for low temperature PhaZ-based technologies, or in environments with high sodium and/or calcium presence, like the ocean.

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6. Structural Variants of Extracellular Polyhydroxybutyrate Depolymerase from *Comamonas testosteroni* 31A Impact Degradation Rates[§]

6.1. Abstract

The extracellular polyhydroxybutyrate (PHB) depolymerase from *Comamonas testosteroni* 31A ($PhaZ_{Cte}$) has been previously characterized showing potential for the development of PhaZ-based technologies. Structural variants of this enzyme were designed and expressed heterologously to investigate how the inclusion or removal of the linker (L) and substrate binding (B) domains, and the inclusion of Soc (S), the small outer capsid protein of bacteriophage T4, impacted the kinetics and stability of $PhaZ_{Cte}$. The degradation activity of the variants was compared and used to decouple the contribution of each domain. The activity of the variants was assessed under selected testing conditions using PHB film — 1 μg/ml variant at 30 °C, measured by spectrophotometric detection of 3HB formation and mass loss of the film. The variants with catalytic (C) domain alone and CL did not show activity; however, hydrolyzing activity could be observed for both variants when tested at much higher concentrations on PHB plates. The addition of S to the wild-type enzyme (SCLB) decreased activity by approximately 20%, while for CLS the replacement of B resulted in approximately 70% decrease in activity — however, it still

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showed superior activity to CL. Overall, the variants retained high stability when stored at -20 °C, 4 °C, room temperature, and 37 °C for 1 and 5 weeks. Storage at 37 °C for 5 weeks led to the largest decrease in activity (by approximately 30%), while no activity loss was detected for storage at -20 °C.

Keywords: *Comamonas testosteroni* 31A, extracellular PHB depolymerases (PhaZ), poly(3-hydroxybutyrate) (PHB), polymer enzymatic degradation, small outer capsid protein (Soc), structural enzyme variants.

6.2. Introduction

Extracellular polyhydroxyalkanoate depolymerases (PhaZs) are broadly studied carboxylesterases based on their capability to break down poly(3-hydroxybutyrate) (PHB) and other polyhydroxyalkanoates (PHAs) [1]. PHAs are a class of natural biodegradable polyesters with large potential to replace some synthetic polymers contributing to the current plastic waste accumulation crisis [2]. The main structural components of extracellular PhaZs consist of a signal peptide (SP) (22–58 amino acid sequence that allows secretion of the mature protein and is cleaved off by signal peptidases), an N-terminal catalytic domain (Cat; C) — involved in the hydrolysis of the ester bonds of PHA by a catalytic triad which consists of serine (Ser), aspartic acid (Asp), and histidine (His) residues — a linker domain (link; L) — approximately 40 amino acids long; provides proper distance and flexibility to the domains of the enzyme — and a C-terminal substrate binding domain (SBD; B) — 40 to 60 amino acids long and responsible for binding the enzyme to the polymer [1,3]. In a few cases (e.g. PhaZs from *Alcaligenes faecalis* AE122,

Pseudomonas stutzeri, *Marinobacter* sp. NK-1, *Bacillus* sp. NRRL B-14911, and *Shewanella* sp. JKCM-AJ6,1α), two SBDs have been reported [4-8].

Several investigations based on fusion proteins have been conducted to study the role of the different PhaZ domains, with special attention to the SBD [1,3]. Most of the examples involved fusing tag proteins to SBDs and were used to identify its function [9-12]: the capacity of the SBD to bind only to solid phase PHB (and not to amorphous emulsified PHB in liquid phase), and that, in some cases, the presence of two SBDs in a single PhaZ is due to weak binding affinities of each SBD [5]. These findings and employing such modified proteins have been important to understand PhaZs, and have led to developing applications such as micropatterning to study protein-protein interactions or immunoassays through protein immobilization on PHB using SBD fusion proteins [13,14].

Functional domain analyses and production of fusion proteins have not been carried out for all reported PhaZs. Among those is the extracellular PhaZ from the soil strain *Comamonas testosteroni* 31A (formerly referred to as *Comamonas* sp.), $PhaZ_{Cte}$, which was first reported and characterized by Jendrossek et al. in 1993 [15] with its *phaZ* sequence made available in 1995 [16]. $PhaZ_{Cte}$, is 53 kDa with and 50 kDa without its SP, and is able to degrade PHB to its monomer at temperatures of 4–58 °C (with optimum at 29–35 °C) and pH 4.8–10.6 (with optimum at pH 9.4 in 0.1 M Tris-HC1 buffer) [1,16]. This enzyme is resistant to many inhibitors, such as phenylmethylsulfonylfluoride, and is reversibly sensitive to dithioerythritol, affecting its disulfide bonds. It is also able to hydrolyze, but at much slower rates, other PHAs such as poly(3-hydroxyvalerate) (PHV) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) [15]. Due to its wide range of applicability and robustness, $PhaZ_{Cte}$ represents a good candidate for the development of

PhaZ-based applications such as polymer waste management and remediation, *in situ* implant removal, and biodegradable biosensors.

To improve performance, such plastic treatment and sensing applications would require improvements and/or structural modifications of PhaZ candidates. Previous studies on modified PhaZs have focused on elucidating the function of the different domains and key residues. However, it is necessary to understand the impact of these domains on both activity and kinetics. Furthermore, for certain applications, like expression in different hosts, modifying the structure of PhaZ enzymes could prove useful by, for example, shortening their structure, improving kinetics, and creating chimeric versions with proteins belonging to the host to facilitate recombination. In such cases it is of interest to explore hybrid versions of PhaZs with proteins other than the typical fusion proteins like maltose-binding protein from *Escherichia coli* (MalE) and glutathione-S-transferase (GST). An example for these applications is phage display, in which fusing a protein to the small outer capsid protein (Soc; S) of bacteriophage T4 [17,18] can facilitate enzyme screening, or to develop phage/PhaZ based biosensors [19].

This study focuses on the exploration of structural variants of $PhaZ_{Cte}$. These variants — which involved removal, inclusion, or replacement of various domains (C, L, B, and S) — enable the decoupling of the binding and hydrolytic processes, the determination of the relevance of various domains for the activity, to investigate if there is potential for integrating $PhaZ_{Cte}$ in a phage expression system, as well as integrity and stability of the enzyme.

6.3. Materials and methods

6.3.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains — specifically, PhaZ-producing *Comamonas testosteroni* 31A (German Collection of Microorganisms and Cell Cultures — Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ) [15,16], cloning strains NEB 5-alpha Competent *E. coli* (Subcloning Efficiency) and *E. coli* ElectroMAX DH10B, and expression strain *E. coli* Rosetta-gami B(DE3) — media, and growth conditions are detailed in Table 4.1. For additional details on growth, such as plating, please see section 4.3.1 Plasmids used in this study are listed in Table 6.1.

Table 6.1. Plasmids used in this study.

Plasmid	Description	Source
pUSCD	T4 integration plasmid for expression of foreign gene attached to C-terminus of Soc and expression of foreign protein on T4 capsid. Integration into T4 genome by *e* and *denV* recombination and selection by completion of lysozyme gene. Ampicillin (Ap) resistance.	Versatile BioSciences
pSL	T4 integration plasmid for expression of foreign gene in T4. Recombination into T4 genome by *e* and *denV* recombination and selection by completion of lysozyme gene. Ap resistance.	Versatile BioSciences
pET-22b(+)	Expression vector that carries N-terminal *pelB* signal sequence for potential periplasmic localization and C-terminal His-tagged for production of proteins in *E. coli*. Ap resistance (*bla* coding sequence 4038-4895).	Novagen

6.3.2. PHB depolymerase variants of PhaZ$_{Cte}$

The six variants of the enzyme PhaZ$_{Cte}$ produced in this study are described in Table 6.2. The structural components used were wild-type Cat (C), wild-type link (L), wild-type SBD (B), consisting of 53 C-terminal amino acids of PhaZ$_{Cte}$, and Soc from bacteriophage

T4 (S). The *phaZ*$_{Cte}$ gene and annotations reported by Jendrossek et al. under GenBank accession number U16275 [1,16] were the basis for domains definition and primer design. All primers used to produce the PhaZ$_{Cte}$ variants are detailed in Table 6.3, while sequencing primers are detailed in APPENDIX C (Table C.1). The variants mCLB, CL, C, CLS, and SCLB contain a methionine residue (m) at the N-terminus — a residue not present in the wild-type version of the enzyme (CLB) after excision of the SP. A His-tag was included at the C-terminus of all variants (built-in the pET-22b(+) sequence by default).

Table 6.2. PhaZ$_{Cte}$ variants.

PhaZ$_{Cte}$ variant	Structural components	Graphical representation
CLB	Cat – link – SBD	
mCLB	M – Cat – link – SBD	
CL	M – Cat – link	
C	M – Cat	
CLS	M – Cat – link – Soc	
SCLB	M – Soc – Cat – link – SBD	

Table 6.3. Primers used for the production of PhaZ*Cte* variants.

Underlined and bold text indicates restriction sites. Double underlined nucleotide was added in primer 10 to correct a frame shift in the CLS variant

Primer number	5' to 3' sequence	PhaZ*Cte* variant
1	CAATTTACGACA**GAATTC**CGCCGTGCCGCTGGGGCAATACAACATT	CLB, pET-22b(+) incorporation
2	GCGATAAACAAT**CTCGAG**GGGGCAGGTACCGATCACGTAGTAGTTGCT	CLB, mCLB, pET-22b(+) incorporation
3	GTTCTCATCTT**GAATTC**CATGGCCGTGCCGCTGGGGCAATA	mCLB, CL, C, CLS, pET-22b(+) incorporation
4	GCACCGTAGC**CTCGAG**CGTGGTGCAGGTGCCCGCAGGA	CL, pET-22b(+) incorporation
5	GCACCGTAGC**CTCGAG**CTGGGTCGGGTTTCCTCCGCCGT	C, pET-22b(+) incorporation
6	CAGGTTC**GGATCC**ACCGCCACCTGGGTCGGGTTTCCTCCGCCG	CLS - CL amplification, pSL incorporation
7	GTTCTCATCTT**CATATG**GCCGTGCCGCTGGGGCAATACAACATT	CLS - CL amplification, pSL incorporation
8	GCACCGTAGC**GGATCC**ATGGCTAGTACTCGCGGTTA	CLS - S amplification, pSL incorporation
9	CAGGTTC**GCGGCCGC**TTAATGATGGTGATGGTGATGACCAGTTACTTTCCACAAATCTTC	CLS - S amplification, pSL incorporation
10	P-GAAACCCGA**C**CCAGGGTGGCGGTGGATC	CLS - site directed mutagenesis to put S in frame with CL
11	GAATACATGTTC**CTCGAG**ACCAGTTACTTTCCACAAATCTTCATTTGCAGCAATCCATTC	CLS, pET-22b(+) incorporation
12	GTTCTCATCTT**GAATTC**ATGGCCGTGCCGCTGGGGCAATACAACATT	SCLB - CLB amplification, pUSCD incorporation
13	GAATACATGTTC**GCGGCCGC**TTAATGATGGTGATGGTGATGGGGGCAGCTGCCGATCA	SCLB - CLB amplification, pUSCD incorporation
14	GTTCTCATCTT**GGATCC**CATGGCTAGTACTCGCGGTTATGTTAATATCAAAACA	SCLB, pET-22b(+) incorporation
15	GAATACATGTTC**CTCGAG**GGGGCAGGTACCGATCACGT	SCLB, pET-22b(+) incorporation

The wild-type *phaZ*$_{Cte}$ gene was obtained from genomic DNA (gDNA) of *C. testosteroni*, extracted using an RNA/DNA purification kit (Norgen Biotek). The wild-type mature enzyme (CLB) and three variant inserts (mCLB, CL, C) were directly obtained by gene amplifications, adding restriction sites *Eco*RI and *Xho*I, through polymerase chain reaction (PCR) using a thermocycler (T100 Thermal Cycler, Bio-Rad) and Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific). The *soc* gene from bacteriophage T4 (S fragment) was previously incorporated in the pUSCD plasmid by the provider (along with 4 glycine residues between S and the *Eco*RI site). For the SCLB variant, the CLB fragment was incorporated into pUSCD using primers 12 and 13 containing restriction sites *Eco*RI and *Not*I. SCLB amplification was then performed with primers 14 and 15, adding restriction sites *Bam*HI and *Xho*I. In the case of the CLS variant, the CL fragment was amplified with primers 6 and 7, adding restriction sites *Bam*HI (along with 4 glycine residues) and *Nde*I, while the S fragment was amplified with primers 8 and 9, adding restriction sites *Bam*HI and *Not*I. The two fragments were incorporated into the pSL plasmid, and site-directed mutagenesis was then performed to correct a frame shift for S. Finally, an amplification was performed with primers 3 and 11 of the whole CLS insert adding restriction sites *Eco*RI and *Xho*I. Restriction digestions — using restriction endonucleases *Eco*RI, *Xho*I, *Bam*HI, *Nde*I, or *Not*I (New England Biolabs and Promega), depending on the variant produced — and ligations were performed to obtain the various inserts. The constructs of the variants were then cloned by incorporation into the pET-22b(+) expression vector. For cloning details please see section 4.3.2. All the variant constructs were confirmed by sequencing (ABI 3730 DNA sequencer, Applied Biosystems).

6.3.3. Expression and purification of PhaZ*Cte* variants

For expression and purification details please see section 4.3.4. Briefly, inductions were performed with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubation was done overnight at 15 °C. During purification, the concentration of imidazole used in the equilibration solutions, sample application, and wash steps was 50 mM. After elution, buffer exchange to 0.1 M potassium phosphate pH 7.4 was done with Amicon Ultra 0.5 mL filters, molecular weight cut off (MWCO): 30K for CLB, mCLB, CL, CLS, SCLB, and 10K for C (Millipore) (only these samples were used for all assays). The remainder of the elution was dialyzed (Slide-A-Lyzer MINI Dialysis Devices, 20K MWCO, Thermo Scientific) to 0.1 M potassium phosphate pH 7.4 as well. Aliquots of the PhaZ*Cte* variants after buffer exchange were used fresh or after storage at -20 °C, 4 °C, room temperature (RT), and 37 °C for stability analyses (without the addition of cryoprotectant or any preservatives).

6.3.4. Characterization of PhaZ*Cte* variants

Fractions containing PhaZ*Cte* variants pre- and post-purification, and a Broad-Range protein standard ladder (6.5–210 kD) (Bio-Rad) were treated for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, based on the methods described by Laemmli [20]. 2X Laemmli sample buffer (Bio-Rad) was added to each sample before they were boiled at 100 °C for 10 minutes. Volumes of samples loaded in the gels were normalized according to the OD_{600} of the cultures (before and at the end of the induction period) to compare the levels of expression. For post-purification samples, dilutions were made to load approximately 500 ng of each variant. The samples were then

run in 12% polyacrylamide gels (Bio-Rad) for 40 minutes at constant voltage (200 V). The gels were washed three times with Milli-Q water for 10 minutes each and stained with PageBlue protein staining solution (ThermoFisher Scientific) for 1 hour under gentle agitation. Finally, the gels were washed with Milli-Q water. Images of the gels were acquired under UV exposure (AlphaImager EC, Alpha Innotech) or with a regular camera. Total protein concentration of PhaZ$_{Cte}$ variants was measured after buffer exchange with Bradford Protein Assay (microassay procedure, Bio-Rad) using a spectrophotometer (Biochrom, Ultrospec 50) and a bovine serum albumin standard.

6.3.5. PHB depolymerase activity

PHB depolymerase activity was assessed using PHB films in two ways: UV-spectrophotometry and mass loss. PHB films approximately 40 µm thick [21] were solution cast at 140 °C from PHB pellets (BRS Bulk Bio-pellets, Bulk Reef Supply, Golden Valley, USA) dissolved in glacial acetic acid (Fisher Scientific), as previously described [21]. The PHB films were cut into 1.3 cm × 1.3 cm pieces for activity assessment, washed with sterile Milli-Q water overnight three times and dried in an oven at 50 °C. PhaZ$_{Cte}$ variants were diluted to a concentration of 1 µg/ml in 0.1 M potassium phosphate pH 7.4 to obtain the enzyme solutions to be tested; this concentration was determined through preliminary experiments to avoid enzyme saturation or inhibition (see APPENDIX C, Figure C.1).

For the UV-spectrophotometry method, a piece of PHB film was exposed to 1.25 mL of enzyme solution in a quartz cuvette (Hellma). Optical density at 210 nm (OD_{210}) was measured using a spectrophotometer (Ultrospec 50, Biochrom) to detect the formation of water soluble detectable products (monomers and dimers of PHB) as the PHB film

degradation proceeded — based on the method by Mukai et al. [22]. A Thermostatted 5-Cell Changer (Biochrom) was used to perform the experiments at 30 °C in parallel mode with measurements performed at 15 s intervals over 180 min. For each sample, the corresponding enzymatic solution was used as reference. Kinetic assays were done with a negative control (PHB film with buffer), fresh PhaZ$_{Cte}$ variants, and stored at -20 °C, 4 °C, RT, and 37 °C for 1 and 5 weeks. All conditions were tested in three or four replicates. A calibration curve with (R)-3-Hydroxybutyric acid (3HB) (Sigma-Aldrich, ≥ 98.0% (T)), and the corresponding OD_{210} values, was performed at 30 °C to determine relative specific activity of the variants.

For mass loss, the individual pieces of dry PHB film were weighed (Dual Range XS105, Mettler Toledo) before exposure to PhaZ$_{Cte}$ variants in the UV-spectrophotometry method. Right after the 180 min exposure to the enzyme, the films were washed two times with sterile Milli-Q water, dried at 50 °C for at least 48 h, and weighed again. Activity was reported as mass loss per surface area of film (3.38 cm^2, thickness was assumed negligible) per enzyme concentration after 3 h of exposure to the enzymatic solutions.

An additional qualitative activity test was carried out using double-layer mineral medium agar plates supplemented with PHB powder (DSMZ list of recommended media for microorganisms, medium 474: 20 ml first layer, 10 ml second layer, described in Chapter 3, section 3.3.4). The plates were pierced to produce cylindrical wells for the deposition of samples. In one set of duplicate plates, 100 µl of PhaZ$_{Cte}$ variants were dispensed at a concentration of 10 µg/ml. Another set was done for CL and C variants at 20, 50, and 100 µg/ml, twice per plate. Plates were incubated at 30 °C and pictures were taken throughout 1 week of incubation.

6.3.6. Statistical analysis

Activity data was reported as mean ± standard deviation (SD). As part of the experimental design and due to the small sample sizes, normal distribution and equal variances were assumed; if more tests were conducted, normality and homoscedasticity would be expected. However, Shapiro-Wilk (for normality, cannot be tested for $n < 3$) and Brown-Forsythe (for equal variances) test results are reported in APPENDIX C, section C.3 for groups that did not meet the normal distribution and equal variance conditions.

One-way analysis of variance (ANOVA) was used to analyze activity data of the PhaZ$_{Cte}$ variants (from UV- spectrophotometry and mass loss), and each variant across storage conditions. Statistically significant differences from ANOVA were followed by Tukey's multiple comparisons test for PhaZ$_{Cte}$ variants and by Dunnett's multiple comparisons test for each fresh variant with the different storage conditions. For one case of PhaZ$_{Cte}$ variants comparison (UV- spectrophotometry data over the 3 h time range), a two-tailed t-test was carried out for direct comparison and it is reported in APPENDIX C, section C.3.

All analyses were done with GraphPad Prism (version 8.0.0), and the level of significance was set at $p < 0.05$.

6.4. Results and discussion

6.4.1. Design, expression and purification of PhaZ*Cte* variants

The wild-type sequence of the mature *phaZ*$_{Cte}$ was sequenced and confirmed from four colonies of *C. testosteroni* 31A. As can be seen in Figure 6.1, six nucleotides differed between the gene sequence reported by Jendrossek et al. [16] (Genbank U16275) from the original isolate and the sequencing results from our strain. Five of these changes led to three amino acid differences (T42I, T457N, A458G) and one to the same amino acid residue (P110P) (position numbering starts from the first amino acid of the mature peptide). The change in residue T42I — from polar threonine to non-polar isoleucine — was located in Cat, but did not affect the key amino acids of the active site (an oxyanion His, and Ser, Asp and His residues of the catalytic triad [1]). The changes in residue T457N — from threonine to asparagine — and A458G — from alanine to glycine — were both located in the SBD, all involved small amino acids and could be conservative substitutions [23,24].

A Basic Local Alignment Search Tool (BLAST) [25] search of the mature wild-type gene sequence at hand (with 100% query cover) resulted in 99% identity to the U16275 sequence reported by Jendrossek et al. [16], and in 97% identity to the sequence of *C. testosteroni* YM1004, a marine strain reported by Shinomiya et. al [12] (nucleotide GenBank: AB000508.1, protein GenBank: BAA22882.1; 100% identical to nucleotide GenBank: CP006704.1 and protein GenBank: AIJ45496.1 from *C. testosteroni* TK102). However, when aligning our expected protein sequence to the protein sequences reported by Jendrossek et al. (nucleotide Genbank U16275, protein GenBank: AAA87070.1), and by Shinomiya et al., both comparisons were approximately 99% identical. For the sequence

reported by Shinomiya et al., the aforementioned amino acid differences (I42I, N457N, G458G) were not present, although seven other differences were found (G128A, T334N, G368S, T382P, Y384S, N394S, S398N). It is possible that the *C. testosteroni* 31A strain (in the taxonomic sense [26]) has undergone changes over time, since the sampling date registers before November 1991 (DSMZ website).

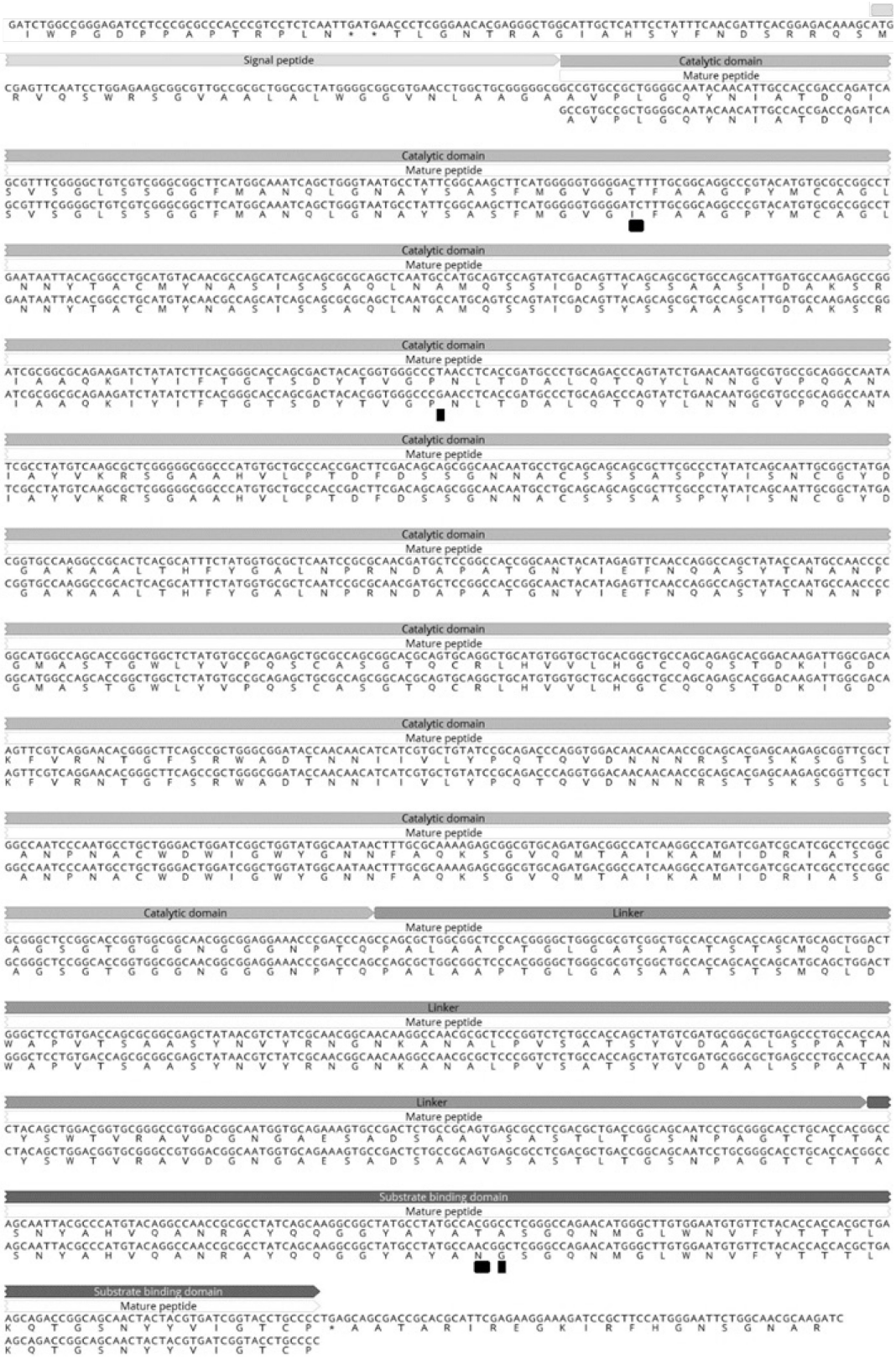

Figure 6.1. PhaZ$_{Cte}$ nucleotide sequence alignment between reported gene in Genbank U16275 and mature peptide sequenced in this study.

Nucleotides with black box underneath show differences.

Following DNA sequencing, the translated mature protein sequence was analyzed with a solubility predictor (PROSO II, [27]) for heterologous expression in *E. coli*. The solubility score of the wild-type PhaZ was 0.504 [28], which classifies it as insoluble (default PROSO II threshold: 0.6). In addition, the protein sequence contains 10 cysteine residues, some of which likely form disulfide bonds — consistent with the sensitivity of extracellular PhaZs to dithiothreitol (DTT) [1]. This is also supported by results from the disulphide bond predictor DiANNA [29-32] which predicts the presence of disulfide bridges at cysteine positions: 50 – 235, 59 – 433, 154 – 226, 165 – 220, and 289 – 487. The predicted insolubility and disulfide bonds from the wild-type protein sequence led us to select the Rosetta-gami B(DE3) *E. coli* platform (designed to enhance the formation of protein disulfide bonds), the plasmid pET-22b(+) (which contains the N-terminal peIB leader sequence for periplasm localization of the heterologous protein — a more favourable environment for disulfide bond formation), and to perform induction with 1 mM IPTG at 15 °C overnight (to reduce the formation of inclusion bodies) for the expression of recombinant PhaZ$_{Cte}$ and its variants. An additional advantage of using Rosetta-gami is that this strain contains the plasmid pRARE that supplies it with tRNAs for five rare codons, among them AUA, AGG, and CCC present 1, 2, and 6 times, respectively, in the mature PhaZ$_{Cte}$ sequence.

All PhaZ$_{Cte}$ variants were successfully expressed using the strategy described above. Figure 6.2 shows post-extraction, soluble and insoluble fractions of induced and non-induced cultures for five of the PhaZ$_{Cte}$ variants produced (mCLB size and expression levels were very similar to CLB — data not shown). The wild-type CLB (lanes 1 to 4) shows high levels of expression, with most of the enzyme present in the induced soluble

fraction, and a smaller percentage in the insoluble induced fraction. These observations also apply to the smaller CL (lanes 5 to 8) and C (lanes 9 to 12) variants. In the case of the CLS (lanes 13 to 16) and SCLB (lanes 17 to 20) variants, expression was only markedly visible in the insoluble induced fractions, with a reduction of expression levels for CLS. There was no apparent leaky expression before induction for any of the analyzed variants.

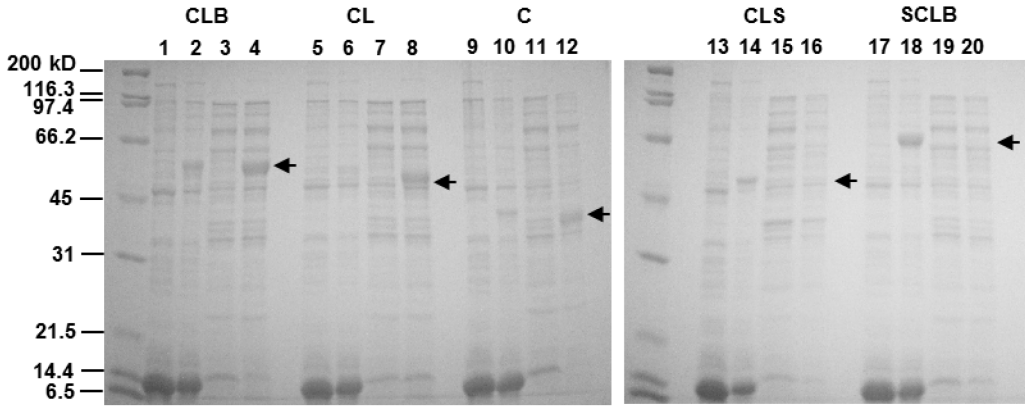

Figure 6.2. Induced and non-induced soluble and insoluble fractions of PhaZ$_{Cte}$ variants.

The loading volumes were normalized according to the OD_{600} reached by the cultures before induction and at the end of the induction period. Variants distribution: CLB lanes 1 to 4, CL lanes 5 to 8, C lanes 9 to 12, CLS lanes 13 to 16, SCLB lanes 17 to 20. The first two lanes for each variant indicate insoluble fractions and the second two soluble fractions. All odd numbered lanes are from cultures before induction and even numbered lanes after induction. Arrows show approximate locations of variants.

Table 6.4 shows theoretical and measured characteristics for the variants. The theoretical enzymes molecular weights (Mw) and isoelectric points (pI) were calculated using the Compute pI/Mw tool from the ExPASy Bioinformatics Resources Portal (SIB Swiss Institute of Bioinformatics) [33,34]. Also, PROSO II [27] was used to predict the solubility of the expressed variants, including amino acids from the design — originating

from the pET-22b(+) plasmid following the signal peptidase cleavage site, restriction sites, and His-tag; all variants were predicted to be insoluble. Finally, the table shows the concentration of the fractions used for dilutions (purified and recovered using buffer exchange columns), and the approximate yield (after purification and buffer exchange) for all variants obtained from 1 L of *E. coli* Rosetta-gami B(DE3) cultures. The yield was high for CLB, mCLB, CL and C (in the range of 6.5 to 9.2 mg), medium for SCLB (2.6 mg), and low for CLS (0.3 mg). In any case, the expression strategy led to enough PhaZ$_{Cte}$ variants in the soluble fractions of cell lysates for analyses. The predicted solubility factors were similar for CLB, mCLB, SCLB, and CL, while those for CLS and C were lower (more soluble); but this parameter and pI do not explain the variations in expression. Partial or complete misfolding, caused by perturbation in residue-residue interactions due to the presence of the new protein sequence, is often encountered in fusion or chimeric proteins, especially when replacing an enzyme's domain as unpredicted alterations in structure or function can be produced [35]. Since SCLB and CLS fall in these categories, this could at least partially explain their lower yield and insolubility (Figure 6.2).

Table 6.4. Theoretical and measured characteristics of PhaZ$_{Cte}$ variants.

1–2: theoretical Mw and pI calculated with Compute pI/Mw tool from the ExPASy Bioformatics Resources Portal (SIB Swiss Institute of Bioinformatics) [33,34,36]; 3: predicted solubility score calculated with PROSO II [27,28].

Characteristic	Variant					
	CLB	**mCLB**	**SCLB**	**CLS**	**CL**	**C**
1. Theoretical Mw [Da]	52824.95	52956.14	62358.62	56587.26	47172.82	37605.44
2. Theoretical pI	6.57	6.57	6.34	6.16	6.17	6.53
3. Predicted solubility score	0.476	0.484	0.531	0.303	0.539	0.346
4. Concentration of fraction used for assays dilutions [µg ml^{-1}]	883 ± 73	957 ± 77	227 ± 6	446 ± 55	386 ± 13	448 ± 16
5. Approximate yield [mg] of variant per liter of *E. coli* Rosetta-gami B(DE3)	9.2 ± 0.05	7.4 ± 0.5	2.6 ± 0.06	0.3 ± 0.03	6.8 ± 0.2	6.5 ± 0.1

SDS-PAGE was performed for each variant using 500 ng of enzyme (Figure 6.3), showing a high level of purity for most variants. Variants SCLB and CLS (columns 3 and 4 respectively) have more of a smeared appearance and additional bands compared to the other variants; this was likely due to stability and solubility issues raised by the insertion of the Soc protein, as mentioned above.

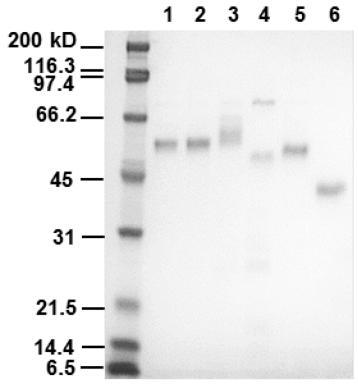

Figure 6.3. Purified PhaZ$_{Cte}$ variants at approximately 500 ng.
1. CLB, 2. m.CLB, 3. SCLB, 4. CLS, 5. CL, 6. C

6.4.2. Kinetics of PhaZ$_{Cte}$ variants

As PhaZ$_{Cte}$ predominantly degrades PHB to its monomer [15,16], its activity can be assessed by monitoring increase in absorbance at 210 nm, a wavelength indicative of the carbonyl groups created by the hydrolysis of PHB to 3HB [22]. Figure 6.4 shows the results of OD_{210} over time generated by PHB film incubated with the negative control (0.1 M potassium phosphate buffer), the wild-type enzyme CLB, as well as the SCL and CLS variants (all at 1 µg/ml). The values were corrected to initiate measurements at 0. The enzymatic activity of the variants tested was determined as the slope of the OD_{210} data between 2000 and 4000 s per variant concentration, and as mass loss per surface area of

PHB film per variant concentration after the 3 h of incubation. The resulting values for activity, R^2 mean values, and relative specific activity between 2000 and 4000 s — using the experimentally determined absorption coefficient of 3HB (59 M^{-1} cm^{-1}) at 210 nm, 30 °C, in 0.1 M potassium phosphate, pH 7.4 — are reported in Table 6.5.

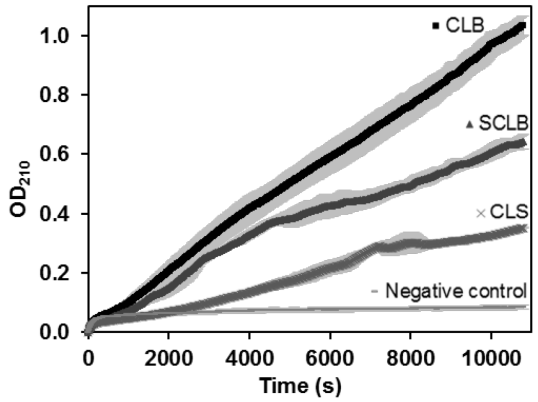

Figure 6.4. Production of the monomer 3HB from PHB film hydrolysis by PhaZ*Cte* variants as a function of time, monitored by OD_{210}.
PHB film was exposed to 1µg/ml of PhaZ*Cte* variants in 0.1 M potassium phosphate buffer, pH 7.4. Results from the wild-type CLB, variants SCLB, CLS, and negative control (PHB film in buffer only) are shown as examples. The lighter gray shade behind the markers is produced by the error bars indicating the SD for each treatment.

Table 6.5. Activity of fresh PhaZ$_{Cte}$ variants.

Superscript letters indicate results of a Tukey's multiple comparison test with 95% confidence. Values sharing superscripts across the same row have statistically equal values.

Result	Variant						Negative control
	CLB	mCLB	SCLB	CLS	CL	C	
Rate per variant concentration [(ΔOD_{210}.ml)/(µg variant.s)]	$1.1 \times 10^{-4} \pm 1 \times 10^{-5}$ ^{ab}	$1.09 \times 10^{-4} \pm 6 \times 10^{-6}$ ^{ab}	$8.6 \times 10^{-5} \pm 2 \times 10^{-5}$ ^{a}	$3.1 \times 10^{-5} \pm 6 \times 10^{-6}$ ^{c}	$8.7 \times 10^{-6} \pm 8 \times 10^{-7}$ ^{d}	$5.5 \times 10^{-6} \pm 5 \times 10^{-7}$ ^{d}	$5.0 \times 10^{-6} \pm 2 \times 10^{-6}$ ^{d}
n	4	4	3	3	4	4	4
R^2	0.998 ± 0.003	0.994 ± 0.005	0.976 ± 0.004	0.995 ± 0.002	0.981 ± 0.005	0.922 ± 0.03	0.899 ± 0.04
Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	$5.5 \times 10^{-2} \pm 7 \times 10^{-3}$	$5.7 \times 10^{-2} \pm 3 \times 10^{-3}$	$4.5 \times 10^{-2} \pm 1 \times 10^{-2}$	$1.6 \times 10^{-2} \pm 3 \times 10^{-3}$	$4.5 \times 10^{-3} \pm 4 \times 10^{-4}$	$2.9 \times 10^{-3} \pm 3 \times 10^{-4}$	$2.6 \times 10^{-3} \pm 9 \times 10^{-4}$
Mass loss per surface area per variant concentration at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.33 ± 0.02 ^{a}	0.32 ± 0.01 ^{a}	0.28 ± 0.01 ^{b}	0.099 ± 0.002 ^{c}	0.02 ± 0.002 ^{d}	0.01 ± 0.003 ^{d}	0.01 ± 0.004 ^{d}
n	4	4	3	2	4	4	4

The variants statistical analysis showed that for the rate per variant concentration [(ΔOD_{210}.ml)/(µg variant.s)] and mass loss per surface area of film per variant concentration after 3 h [(mg PHB.ml)/(µg variant.cm^2)], there were statistically significant differences between groups as determined by one-way ANOVA ($F(6,19) = 114.4$, $p < 0.0001$ and $F(6,18) = 755.4$, $p < 0.0001$, respectively). Variants CLB and mCLB displayed the highest activities and were statistically equal — with values of $1.1 \times 10^{-4} \pm 1 \times 10^{-5}$ and $1.09 \times 10^{-4} \pm 6 \times 10^{-6}$ (ΔOD_{210}.ml)/(µg variant.s) for monomer production and 0.33 ± 0.02 and 0.32 ± 0.01 (mg PHB.ml)/(µg variant.cm^2), respectively. This confirms the insertion of the methionine residue in mCLB did not affect the degradation behavior. The next variant with highest activity was SCLB ($8.6 \times 10^{-5} \pm 2 \times 10^{-5}$ (ΔOD_{210}.ml)/(µg variant.s) and 0.28 ± 0.01 (mg PHB.ml)/(µg variant.cm^2)). This indicates that the presence of the Soc protein on the catalytic site, while slightly reducing activity — SCLB was found to be statistically significantly lower for the mass loss result — did not lead to an important loss of activity of the enzyme. Next, the CLS variant showed lower activity ($3.1 \times 10^{-5} \pm 6 \times 10^{-6}$ (ΔOD_{210}.ml)/(µg variant.s) and 0.099 ± 0.002 (mg PHB.ml)/(µg variant.cm^2)) than CLB, mCLB, and SCLB (all with $p < 0.0001$). This was expected since the SBD was replaced by a protein showing no specific affinity to binding PHB. Finally, the CL and C variants had the lowest activities ($8.7 \times 10^{-6} \pm 8 \times 10^{-7}$ and $5.5 \times 10^{-6} \pm 5 \times 10^{-7}$ (ΔOD_{210}.ml)/(µg variant.s), 0.02 ± 0.002 and 0.01 ± 0.003 (mg PHB.ml)/(µg variant.cm^2), respectively), and neither showed statistically significant degradation compared to the negative control. The fact that CLS retains activity while none was detectable from CL suggests that the presence of Soc in CLS, although playing no functional role, could impact the conformation of the catalytic domain.

Fusion proteins and variants have been explored in previous investigations to study the functionality of different PHB depolymerases and their domains [1,3]. Some of these studies include fusing MalE with the SBD of PhaZ5 from *Paucimonas lemoignei* [11], and fusing the affinity tag GST to the SBD of *Comamonas acidovorans* [9,10], *Alcaligenes faecalis* [10], *P. stutzeri* [10], and *Comamonas testosteroni* YM1004 [12]. In all cases the conclusion was that the SBD sequences were sufficient and necessary for binding to PHB. Other studies have built a wider array of modified proteins by fusing thioredoxin (TrxA) and GST with several domains from PhaZs for which two SBDs have been identified (SBDI and SBDII): PhaZ from *Bacillus* sp. NRRL B-14911, for which two new types of linker domains were reported (linkI and linkII), with TrxA fused to linkI, linkII, SBDI, SBDII, and linkII-SBDI-II — in addition, they also successfully produced non-fused variants of the N-terminal (Cat domain) and C-terminal domain (both link and both SBD domains), and wild-type with a linkI deletion — [8]; PhaZ from *P. stutzeri*, with GST fused to Cat-link, SBDI, SBDII, and SBDII-I. where only the last three were able to bind to PHB [7]; and PhaZ from *Marinobacter* sp. NK-1 ($PhaZ_{Msp}$), with GST fused to Cat-link-SBDII-I, Cat-link-SBDII, Cat-link, Cat, SBDII-I, SBDI, SBDII, link-SBDII-I, link-SBDII, and link [5].

This last study on $PhaZ_{Msp}$ involved analysis of adsorption to PHB and of specific hydrolyzing activity (by turbidity changes of substrate suspensions at 37 °C — with the soluble ester *p*-nitrophenyl butyrate (PNPB), denatured and emulsified PHB). Among some of their main findings, Kasuya et al. [5] saw that fusion proteins lacking SBDs had very low hydrolytic activity towards denatured PHB, and that the SBDs bound to denatured PHB (solid) but not to emulsified PHB (amorphous fluid). This indicated that the SBDs

needed rigidity to bind to the substrate. The authors also hypothesized that the presence of two SBDs was required to overcome their respective weak binding affinity [5]. Although PhaZ$_{Cte}$ contains only one SBD, the variants used in this study have some similarities with Kasuya's study. These include a reduction of activity for the GST fused wild-type variant compared to wild-type, which was also observed for SCLB compared to CLB. This could be caused by a slight conformational change of the catalytic site due to the fusion protein or to partial steric hindrance resulting from the presence of the fused protein near the catalytic site. Another similarity was found in the significant decrease in PHB hydrolyzing activity for the C variant (Cat alone), also observed with PhaZ$_{Msp}$ [5], PhaZ from *Bacillus megaterium* N-18-25-9 [37], and PhaZ from *Bacillus* sp. NRRL B-149 [8] (in most cases not affecting soluble substrates hydrolyzing activity, except for PhaZ$_{Msp}$). One more common aspect was the importance of the presence of SBD to retain high hydrolytic activity, also observed in a variants study of PhaZ from *Cupriavidus* sp. (formerly *Alcaligenes faecalis* T1 — see APPENDIX A, section A.4) where trimer and PHB hydrolyzing activities were the same for wild-type, a variant with a threonine-rich linker domain replacing the original fibronectin type III (Fn3) domain, and another variant with two linker domains instead of one [38]. On the other hand, variants with Cat alone, Cat-SBD, Cat-SBD-link, and trypsin digested wild-type had no PHB hydrolyzing activity but were able to hydrolyze trimers [38]. Overall, the SBD seems to play a much more important role in hydrolyzing rigid solid polymeric substrates than soluble substrates.

Even though previous studies have found that the SBD is determinant to get high activity, it is important to establish domain behaviour for the PhaZ of interest, as there is even a case of a wild-type PhaZ from *Shewanella* sp. JKCM-AJ6,1α (with a sequence that

revealed the presence of two SBDs) that lost its SBDs, with the truncated version having a degradation advantage under marine conditions due to reduced self-inhibition (despite having less binding ability) compared to a recombinant version with the SBDs [6].

6.4.3. Qualitative PHB plate assay

While no significant hydrolytic activity was detected for variants CL and C, observations of halos formation on PHB plates suggested that they retained activity (Figure 6.5, rows 2 and 3). To qualitatively assess their potential for PHB degradation, a concentration of 10 μg/ml of each PhaZ$_{Cte}$ variant was tested in PHB plates (Figure 6.5, row 1). Their respective degradation was displayed as halos around the wells where they were deposited. As expected, degradation halos grew over the period tested for variants CLB, mCLB, SCLB, CLS, and CL. The relative sizes of the halos were moderately consistent with the results from the kinetic and mass measurements assays. Degradation by variants CL and C, which had limited or no activity during the previous assays, was further investigated using higher concentrations of enzymes (20, 50 and 100 μg/ml) (rows 2 and 3 correspondingly). Both variants showed visible activity, in the case of CL for all three concentrations tested (even after one day of incubation), while for C activity could be detected at 50 and 100 μg/ml (this last concentration showing halo after one day of incubation). Compared to the PHB film results, very high concentrations of CL and C variants and longer incubation periods were necessary for activity to be observed, and the available surface area of the PHB powder suspended in the agar matrix could be larger than that of PHB film, but the substrate remains an insoluble polyester. The halos resulting from degradation by C showed less intensity and were less defined than the ones observed with

the other variants, which seems to support that link and SBD help to maintain an optimum conformation of Cat.

Jendrossek and Handrick have stated that shortened PhaZs lacking the SBD lose the ability to bind and hydrolyze denatured PHB, but they can still hydrolyze soluble esters like *p*-nitrophenylesters [1]. The subsequent study by Kasuya et al., conducted with variants from PhaZ$_{Msp}$ (with substrate in suspension), confirmed the ability of Cat alone and fused to GST to hydrolyze a soluble ester, in this case PNPB [5]. However, the study also demonstrated that these variants could also hydrolyze denatured PHB, although at much reduced rates (exhibiting 28 times less specific activity compared to the wild-type version, and 4 times less than the variant containing GST and link domain) [5]. The fact that Cat alone, of at least some PhaZs, has diminished but remaining activity — corroborated in our plate experiments (Figure 6.5, row 3) — could be attributed to random collision between the truncated enzyme and the PHB substrate following diffusion, which has been described for other surface active enzymes such as residual collagenolytic activity of truncated *Clostridium histolyticum* collagenase enzymes without a collagen-binding domain [39]. It is also possible that the agar matrix facilitates the interaction of the enzyme with the PHB particles (rather than enzyme in a free-flowing solution), but halos would not be observed if the C and CL variants were not able to hydrolyze PHB.

Also, higher activity of PhaZ$_{Cte}$ CL compared to C is analogous to findings of the PhaZ$_{Msp}$ variant study (in which GST-CL had greater activity than GST-C) [5]. Kasuya et al. attributed this difference to the possibility that the cadherine-type linker domain could aid in maintaining a catalytically active conformation of Cat, as link was not able to bind to PHB [5]. For PhaZ$_{Msp}$ link is cadherin-type [5], while link in PhaZ$_{Cte}$ is Fn3 [16]; this

latter type is also found in PhaZs from strains like *Alcaligenes faecalis* AE122, *Cupriavidus* sp., and *Ralstonia* sp. [1,4,16,40]. Aside from providing flexibility between the SBD and Cat, the function of link, in general, and of its conserved amino acids has been described as unknown [1,4,16,40]. It has been hypothesized that the repetitive hydroxylated amino acids in the Fn3 link (similar in this feature to the threonine-rich link regions of PhaZs from *P. lemoignei*) could interact with semi-crystalline PHB — in the case of the studied PHB films, approximately 70% crystallinity for the fabrication method employed [21], and unknown for the suspended PHB powder of the plates — to direct it towards the PhaZ active center [41]. In the study investigating variants of *Cupriavidus* sp., it was hypothesized that the hydroxyl clusters of this domain aided the disruption of the crystalline regions of PHB [38]; but for this to be the case, Fn3 link has to be close to Cat, as variants with swapped link and SBD (Cat-SBD-link instead of wild-type Cat-link-SBD) and without link (Cat-SBD) lost PHB hydrolyzing activity [38]. In addition, when Fn3 link of *Cupriavidus* sp. was replaced by a threonine-rich link of PhaZ5 from *P. lemoignei*, it displayed the same PHB hydrolyzing activity [38]. Therefore, based on these observations and the comparative reduction in the activity of our CL and C variants, it is plausible that for $PhaZ_{Cte}$ Fn3 link impacts the conformation of Cat to retain greater activity, in addition to acting as a spacer between Cat and SBD.

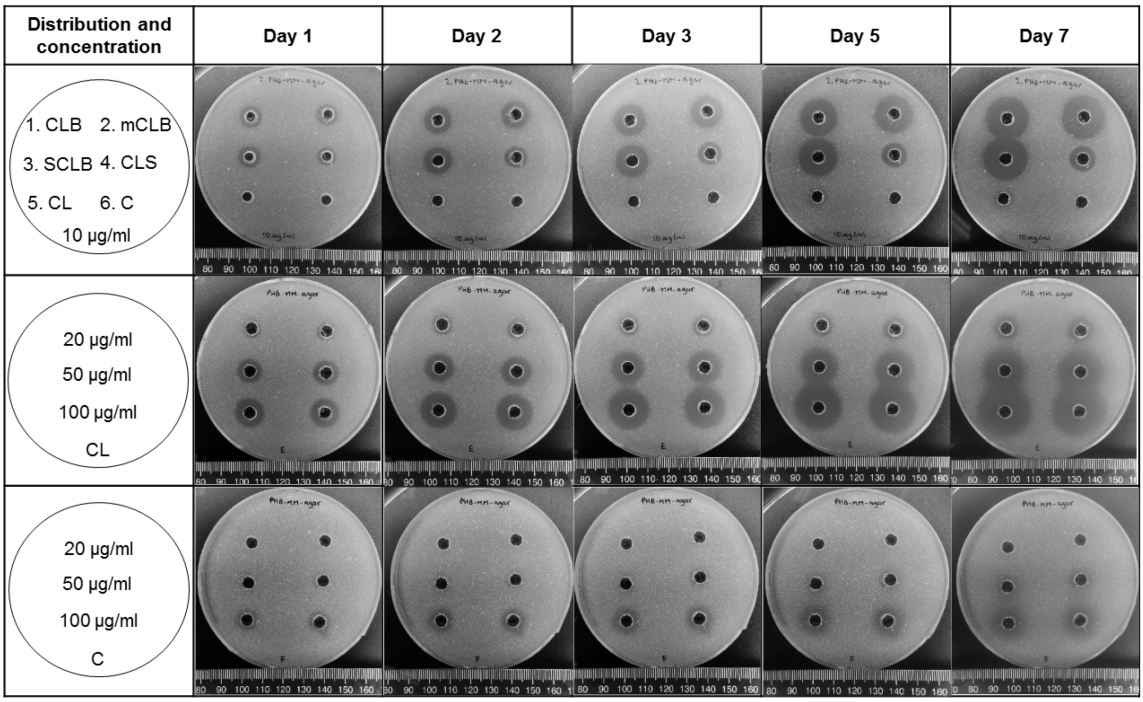

Figure 6.5. Purified PhaZ$_{Cte}$ variants in PHB-mineral medium-agar suspension incubated at 30 °C.

Variants distribution in the first row (all at 10 µg/ml, representative sample): 1. CLB, 2. mCLB, 3. SCLB, 4. CLS, 5. CL, and 6. C. In the second row, variant CL was evaluated in duplicates at concentrations of 20, 50 and 100 µg/ml, and in the third row variant C was evaluated in the same fashion.

6.4.4. Stability of PhaZ$_{Cte}$ variants

The stability of the PhaZ$_{Cte}$ variants — except for CL and C which did not show quantifiable activity in UV- spectrophotometry and mass loss assays — was evaluated under different storage conditions (-20 °C, 4 °C, RT, and 37 °C after 1 and 5 weeks). The results are reported in Figure 6.6 and Figure 6.7 (in more detail in APPENDIX C, Table C.2). In general, the tendency of the variants carrying intact PhaZ$_{Cte}$ (CLB, mCLB, and SCLB) was to show greater degradation throughout all the storage conditions compared to CLS. The first general observation was that, overall, high retention of activity was observed

for all variants across the different storage conditions, but there were differences in the mean values for both activity measurements (confirmed by comparison of activities for all storage conditions by one-way ANOVA analyses for each variant). In the worst cases, activity decreased by roughly 30% for CLB, mCLB, SCLB, and CLS stored at 37 °C for 5 weeks. SCLB showed no significant decrease in rate, however this may be due to large standard deviation in the activity of the fresh enzyme. Freezing at -20 °C appears to be a good storage method as none of the variants had a statistically significant activity decrease after 5 weeks, even if no cryoprotectant was used — however, care must be taken to avoid freeze/thaw cycles. The presence of the heterologous Soc protein in SCLB and CLS did not seem to negatively affect the stability of the enzymes, as there were no statistically significant differences in loss of activity compared to other variants.

Jendrossek et al. reported that PhaZ$_{Cte}$ purified from the wild-type strain (corresponding to CLB in the present study) was stable for weeks when stored at 4 °C and -20 °C, and lost 15% activity when stored at -70 °C and -196 °C [15]. It is likely that partial denaturation of the enzyme occurs for non-optimum storage temperatures — due to freezing/thawing process for -70 °C and -196 °C, and perhaps for RT and 37 °C since at rising temperatures enzymes tend to unfold in a cooperative process (the temperature where the concentration of native enzyme is equal to the concentration of partially denatured enzyme is denominated melting temperature, usually around 60–80 °C in aqueous solutions) [42,43]. The enzyme and its variants (including those containing Soc) still retain high activity even at 37 °C over 5 weeks, which gives them potential for applications over a wide temperature range. Nonetheless, longer-term tests with a broader range of buffers should be conducted to obtain a more complete picture of the stability of PhaZ variants.

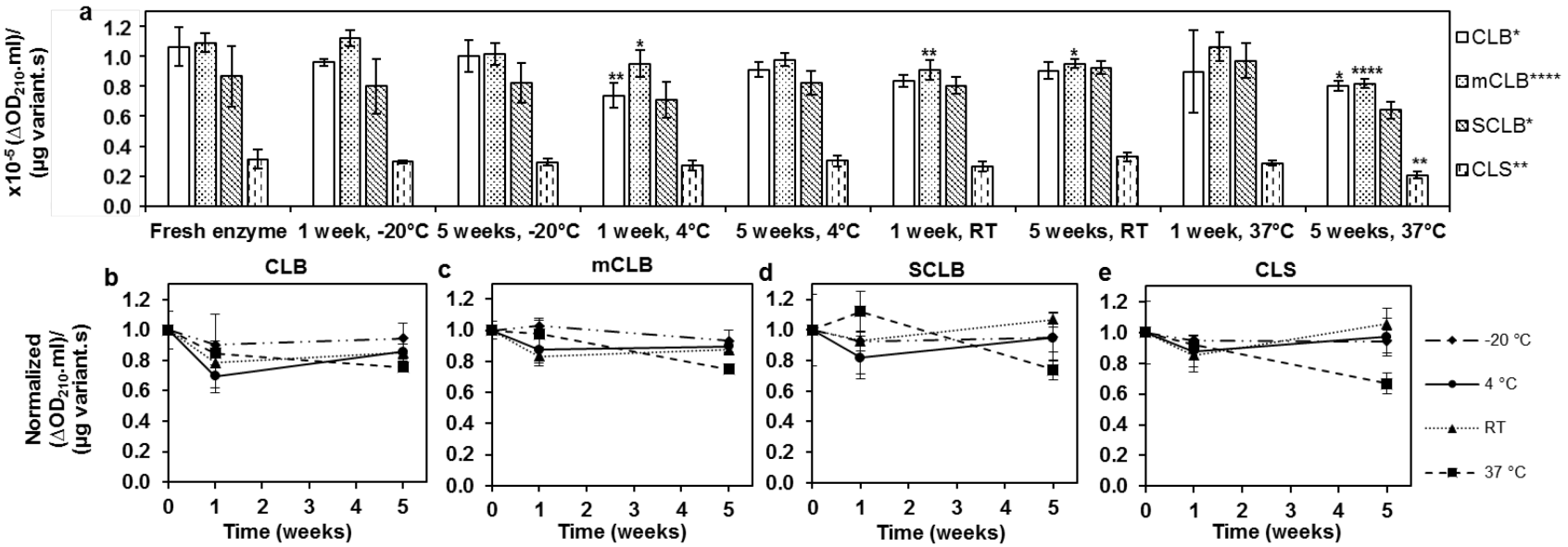

Figure 6.6. Spectrophotometric activity results for PhaZ*Cte* CLB, mCLB, SCLB, and CLS fresh variants and stored at -20 °C, 4 °C, RT, and 37 °C after 1 and 5 weeks.

(a) Rate per variant concentration (ΔOD210.ml)/(µg variant.s) from 2000–4000 s. (b) to (e) Rate data per variant normalized per own fresh value. Data is presented as mean ± SD; in (a) statistical significance levels for ANOVA are indicated next to the name of the variant and for Dunnett's multiple comparisons test for each variant (fresh compared to each storage condition), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (b) to (e) -20 °C longest dashes, 4 °C solid line, RT dots, and 37 °C dashes.

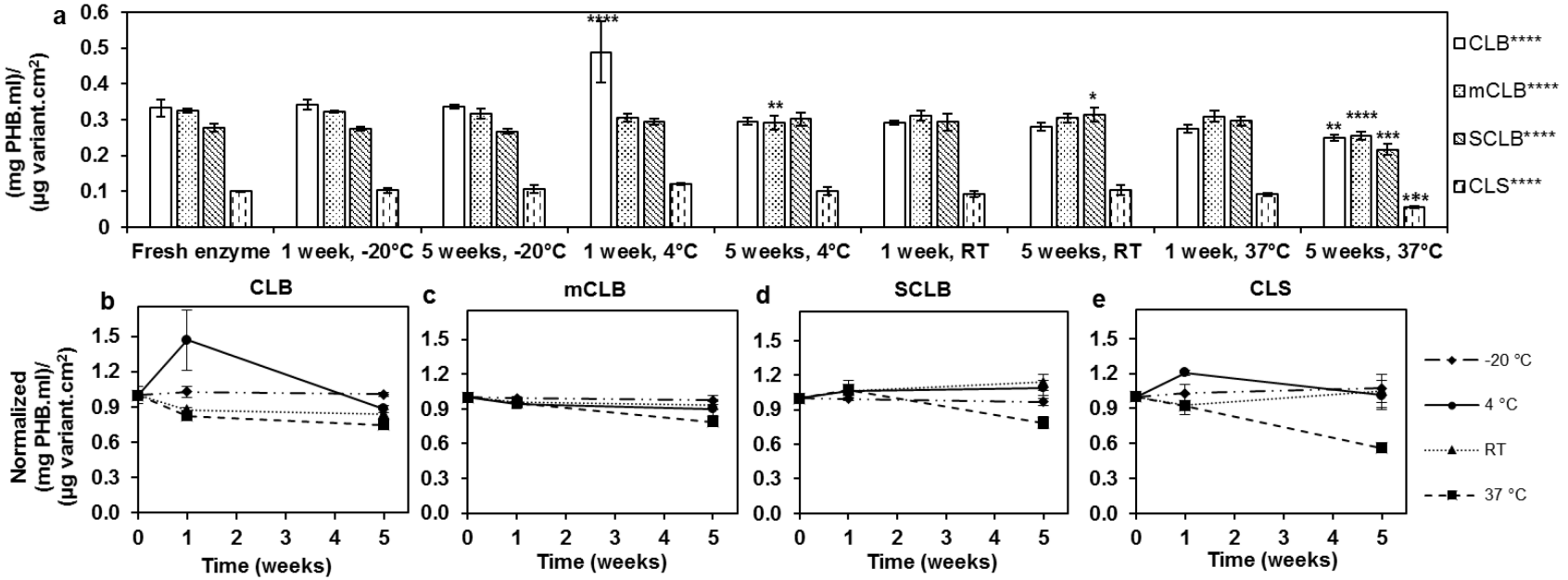

Figure 6.7. Mass activity results for PhaZ$_{Cte}$ CLB, mCLB, SCLB, and CLS fresh variants and stored at -20 °C, 4 °C, RT, and 37 °C after 1 and 5 weeks.

(a) Mass loss per surface area of film per variant concentration after 3 h (mg PHB.ml)/(µg variant.cm2). (b) to (e) Mass loss data per variant normalized per own fresh value. Data is presented as mean ± SD; in (a) statistical significance levels for ANOVA are indicated next to the name of the variant and for Dunnett's multiple comparisons test for each variant (fresh compared to each storage condition), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (b) to (e) -20 °C longest dashes, 4 °C solid line, RT dots, and 37 °C dashes.

6.5. Conclusion

This study of PhaZ$_{Cte}$ variants highlights the roles of the various domains of the enzyme, in its activity and stability under a range of conditions. When investigating the effect of the presence of the native domains, we confirmed that the presence of SBD is determinant to high activity; however, activity — as determined by the rate of production of the monomer, mass loss and visual PHB degradation — was retained for all variants of the enzyme. This includes Cat with and without link. In addition, we have shown that Fn3 link is not restricted to providing space between Cat and SBD; its presence contributed to greater activity (compared to Cat alone) and appeared to support the proper conformation of Cat even in the absence of SBD. The study also provides information on the effects of including fused protein sequences (in this case the Soc protein of bacteriophage T4) to the enzyme. The presence of Soc at the N-terminus of PhaZ$_{Cte}$ reduced hydrolytic activity by approximately 20%, likely caused by reducing the availability of the catalytic site. Interestingly, replacing SBD with Soc only reduced hydrolytic activity by approximately 70%, compared to a greater reduction in activity when SBD was removed without substitution. This suggests the presence of a protein sequence at the SBD location can aid to maintain the conformation of Cat and link, retaining greater activity despite having no specific affinity to the polymer. Finally, we also found that the storage of PhaZ$_{Cte}$ variants in the range of -20–37 °C for up to five weeks resulted in highly retained activity. In the worst case, a 30% reduction was observed after storage at 37 °C, while storage -20 °C led to no loss in activity. This confirms the robustness of this enzyme, which makes it ideal for

many applications requiring PHB degradation. PhaZ$_{Cte}$ and its Soc variants could potentially be used for applications like enzyme-responsive biosensors.

6.6. Acknowledgements

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poly(3-hydroxybutyrate) depolymerase of *Comamonas* sp. and of its structural gene. Canadian Journal of Microbiology 1995;41:160–169.

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7. Summary, Conclusions, and Future Directions

7.1. Summary and conclusions

This thesis explored enzymatic degradation of poly-3-hydroxybutyrate (PHB) by extracellular PHB depolymerases (PhaZs), generating new knowledge on this front with potential for PhaZ-based technologies incorporation. Focus was initially placed on establishing comparisons of bacterial strains from diverse sources with demonstrated and predicted PhaZ activity, moving on to the production and use of five purified recombinant PhaZs, the expression and assessment of PhaZs from *Marinobacter algicola* DG893 ($PhaZ_{Mal}$) which was until now only a putative enzyme, and the construction of modified versions of PhaZs from *Comamonas testosteroni* 31A ($PhaZ_{Cte}$). Specifically, the following conclusions could be generated from the work presented:

Direct comparisons between PHB-degrading microorganisms and excreted PhaZs were addressed in "Polyhydroxybutyrate biodegradation using bacterial strains with demonstrated and predicted PHB depolymerase activity" (Chapter 3). In this study, PHB film degradation by five strains with proven PhaZ activity — *Comamonas testosteroni* 31A, *Cupriavidus* sp., *Paucimonas lemoignei*, *Pseudomonas stutzeri*, and *Ralstonia* sp. — and four strains with predicted PhaZs — *Alteromonas macleodii, Loktanella vestfoldensis, Marinobacter algicola* DG893, and *Oceanibulbus indolifex* Hel45 — was compared under the same controlled laboratory conditions. This helped identify *Ralstonia* sp. as the strain with the highest specific activity and that it was able to preserve activity even at low pH values. Of the strains with putative PhaZs, only *M. algicola* DG893 was able to cause PHB

films mass loss in liquid culture, which hinted that this strain could be able to produce an extracellular PhaZ. This study provides a starting point for the comparison of PhaZ enzymatic activity across bacterial species and strains — especially important as previous studies have typically focused on evaluating a single strain or enzyme in specific conditions — the evaluation of mechanisms, and the assessment of the fate of PHB under certain conditions.

Given the importance of studying purified PhaZs to better understand their mechanisms, develop new technologies, and due to the challenges implied in purifying proteins from their wild-type producing microorganisms (e.g. time-consuming and low yields), we saw the need to establish an efficient method to produce PhaZs. A rationalized platform for the rapid production of recombinant PhaZs was developed and presented in Chapter 4 "Streamlined production, purification, and comparison of recombinant extracellular polyhydroxybutyrate depolymerases". Five active PhaZs from diverse environmental sources (PhaZ$_{Cte}$, PhaZ$_{Mal}$, and PhaZs from *Cupriavidus* sp. (PhaZ$_{Csp}$), *Pseudomonas stutzeri* (PhaZ$_{Pst}$), and *Ralstonia* sp. (PhaZ$_{Rsp}$)) were successfully expressed and recovered. An important aspect of the platform developed involved removing the native signal peptide sequence of PhaZs; this facilitated the recovery of soluble active mature enzymes and avoided the production of insoluble and inactive enzymes. Expression and purity levels varied for each PhaZ, with PhaZ$_{Cte}$ and PhaZ$_{Pst}$ showing highest expression. Degradation activity of the purified PhaZs was initially compared using PHB-agar plates under different pH and temperatures. Findings included PhaZ$_{Mal}$ as the most active enzyme at low temperature, and PhaZs from *Cupriavidus* sp. and *Ralstonia* sp. displaying more activity at low pH. This showed that the PHB-agar method can be used

for rapid evaluations of comparative activity for applications like biosensors or to screen engineered enzyme improvements. The recombinant production platform opens the door to the rapid development and production of recombinant PhaZs — while considering characteristics of PhaZs sequences that might be overlooked in other studies, like solubility, rare codon presence, and disulfide bond formation — and was the basis to produce enzymes and variants studied in Chapter 5 and Chapter 6.

Due to the capacity of *M. algicola* DG893 to degrade PHB demonstrated in Chapter 3, further investigations of its PhaZ were undertaken and reported in Chapter 5 "A novel polyhydroxybutyrate depolymerase from the marine bacterium *Marinobacter algicola* DG893". In this study, media modified to better simulate marine environments were used to determine if they would induce PhaZ activity in the bacterial marine strains with predicted PhaZ activity studied in Chapter 3. Again, it was confirmed that, of all the strains, only *M. algicola* displayed potential PhaZ production. We experimentally validated the predicted $PhaZ_{Mal}$ by producing it heterologously and characterizing its degradation activity with PHB film under several conditions. Comparisons with $PhaZ_{Cte}$ (of soil origin) revealed some similarities, such as the 3HB monomer being the final main degradation product and similar morphological patterns when PHB films underwent degradation equivalent to over 10% of mass loss. However, important differences were also found: 1) $PhaZ_{Mal}$ initially favoured the production of the dimer as a primary product of hydrolysis; 2) significant amounts of trimer were also detected during degradation by $PhaZ_{Mal}$; 3) film surface morphologies were different when PHB was degraded at low levels (< 2.2% mass loss) by either enzymes, with a more pronounced whiskers pattern shown earlier with $PhaZ_{Mal}$; 4) the presence of metal ions like Ca^{2+} and Na^{+} had opposite effects on the two

enzymes, increasing PhaZ$_{Mal}$ activity while reducing that of PhaZ$_{Cte}$ — this was likely due to the enzyme origins (marine versus soil), amino acid sequence, and conformation. In general, PhaZ$_{Mal}$ had slower degradation rates compared to PhaZ$_{Cte}$, but further activity improvement could be achieved by optimizing metal ions concentrations in the medium. Additional exploration of *M. algicola* DG893 and PhaZ$_{Mal}$ could lead to their utilization for low temperature PhaZ-based technologies under pH values close to neutrality, or in environments with high contents of sodium and/or calcium.

Throughout the studies in Chapters 3, 4, and 5, PhaZ$_{Cte}$ showed good potential: demonstrating high activity compared to the other enzymes tested, displaying high yield and purity during heterologous expression, and retaining some activity at low pH (unlike PhaZ$_{Pst}$ and PhaZ$_{Mal}$ that were inactivated) and low temperature. For these reasons, and to better understand its modes of operation, variants of PhaZ$_{Cte}$ were designed, produced and assessed (Chapter 6). These variants involved removal of domains and replacement or addition of a protein domain (the Soc protein from bacteriophage T4) which is not involved in PHB interactions. PHB film degradation kinetics and enzyme stability were studied. Some of the results highlights included: 1) the roles of the various domains, including the fact that the linker domain provides improved stability to the catalytic domain; 2) the impact of the fused Soc protein which only slightly reduced the activity of the full enzyme but also provided conformation stability to the catalytic domain when replacing the native substrate binding domain. The wild-type PhaZ$_{Cte}$ along with its variants were able to retain activity up to approximately 70% when stored at different conditions, including when stored at 37 °C for five weeks. PhaZ$_{Cte}$ and the variants produced could potentially be used for applications like enzyme-responsive biosensors.

Overall, the resulting work led to improved knowledge on PhaZs which can be used in a variety of fields and applications, from environmental remediation to PHB-based sensors and devices. It established clear comparisons between several PHB-degrading bacteria and PhaZs — such studies are scarce in the available literature. In the course of the work, useful methods and techniques were developed that can accelerate future PhaZ research. This included a platform for the rapid development of recombinant PhaZs expression systems — demonstrated with five diverse PhaZs and four variants of $PhaZ_{Cte}$; this platform could be used to produce other PhaZs reported in the literature or novel ones to be discovered — and modifications to the PHB plates screening method — which enabled, for example, widening the pH range tested. These research efforts also led to experimentally validating a protein with predicted PhaZ activity ($PhaZ_{Mal}$), characterizing it, and comparing it to a well described PhaZ — this is a novel addition to the array of PhaZs in the literature, with differences in degradation mechanism. Finally, modifications of $PhaZ_{Cte}$ helped determine the impact of the different enzymatic domains on degradation kinetics and enzyme stability. These contributions add new knowledge on the extracellular PHB degradation front — a field continuously searching for novel and modified PhaZs that can be employed in innovative applications and solutions to problems like plastic waste accumulation in the environment — provide better insight of the performance for some of the studied PhaZs and will facilitate the development of PHB and PhaZ-based technologies.

7.2. Future directions

Several projects can expand from the findings presented in this PhD thesis. Some of them could include:

An exciting avenue to explore is protein engineering. By making use of this powerful tool, some of the PhaZs studied could be modified and improved. This can be achieved by increasing activity, changing substrate specificity, conferring new functions or increasing stability under harsh conditions (e.g. temperature, pH, and solvents). The two central strategies used for protein engineering are directed evolution and rational design. An example could be to increase activity at low temperatures, for which directed evolution has been considered an adequate strategy [1]. Another approach could come from hypotheses — reached by molecular dynamics computational simulations — that cold-adapted enzymes have a more flexible surface and that rigidity outside the catalytic site tunes the enthalpy–entropy balance [2-4]. However, to make use of rational design based on these hypotheses it might be necessary to obtain the crystal structures of PhaZs of interest or at least generate reliable models — both of which have been found to be difficult for PhaZs. The recombinant protein production platform presented in this work has potential to be a powerful tool if a directed evolution approach — e.g. random mutagenesis by error-prone PCR — is selected, or even to screen mutants generated by rational or semi-rational design. This, combined with PHB plate-based screening, would greatly accelerate protein engineering strategies. As an example, Figure 7.1a shows *E. coli* Rosetta-gami B(DE3) colonies displaying halos due to PhaZ$_{Pst}$ expression and secretion on a LB-PHB plate. This can be used as pre-screening, to later perform high-throughput screening of lysates on multi-well plates containing mineral medium-PHB agar on the lid (Figure 7.1b). The PHB plates can be screened at different temperatures, pH values, or they could also be fabricated with other polyesters.

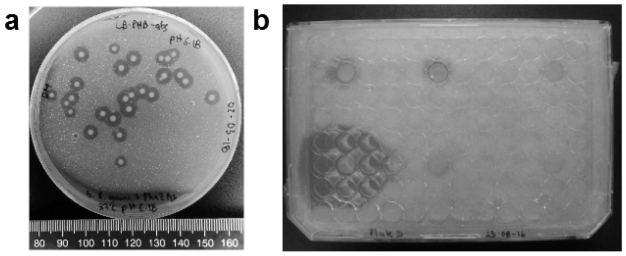

Figure 7.1. Example for PhaZ screening on PHB-agar based media.
(a) *E. coli* Rosetta-gami B(DE3) colonies displaying halos due to PhaZ$_{Pst}$ expression and secretion on LB-PHB plate. (b) High-throughput screening of lysates of *E. coli* Rosetta-gami B(DE3) cultures after expression of PhaZ$_{Pst}$, PhaZ$_{Rsp}$, and PhaZ$_{Csp}$.

Other future avenues of research include the further optimization of media for degradation by PhaZ$_{Mal}$, and the investigation of other polyester substrates to test the specificity of this PhaZ. In addition, it would be interesting to determine if PhaZs from strains with predicted PhaZ activity but that did not display this activity (e.g. *A. macleodii*) could be expressed heterologously and degrade PHB. The PhaZ from *A. macleodii*, unlike *L. vestfoldensis* and *O. indolifex*, had a predicted signal peptide. Even though the strain was not able to display PhaZ activity, it is possible that it requires different medium conditions to the ones explored to be expressed, or that the gene is silenced.

Finally, the PhaZs or PhaZ-producing microorganisms studied could be employed in PHB recycling, 3HB production, or biosensors like the microbial detection-based platform [5], among other applications.

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APPENDIX A: Supplementary Material for Chapter 3

A.1. Additional controls for OD_{600} measurements of strains with elevated growth with PHB film as carbon source

Due to the high OD_{600} values reached by the strains *C. testosteroni, Cupriavidus* sp.*,* and *Ralstonia* sp., more controls were established in addition to the abiotic sample containing PHB film. These controls were performed to determine if growth was aided by remaining media from pre-culture inocula or by trace amounts of acetic acid that could have remained from the PHB film fabrication process (although, we consider that after solvent casting at 140 °C — above the acetic acid boiling point, 118 °C — washing, drying at room temperature for one week, and autoclaving, it is unlikely that a significant amount of acetic acid was left in the film; and this is supported by the lack of evidence of residual solvent through thermogravimetric analysis after fabrication and storage at room temperature for 24 h [1]).

The pre-cultures and inoculation conditions were set up in the same manner as for PHB mass loss experiment with bacterial strains. The first set of negative controls (strain without PHB) consists of the 10% v/v inoculum alone in fresh medium. The second set (strain with acetic acid and without PHB) was the 10% v/v inoculum in addition to 2 µl of acetic acid – equivalent to 1% of the mass of PHB film used in the previous experiment (the maximum amounts of PHB film mass loss detected for the abiotic control treatment were 0.86 ± 0.06 % at time point 48 h and 0.8 ± 0.2 % at time point 96 h). The results in Figure A.1 demonstrate that any OD_{600} contribution that could have been generated from

the remaining media in the inocula (tryptic soy broth for *C. testosteroni*, and nutrient broth for *Cupriavidus* sp., and *Ralstonia* sp.), even with the addition of acetic acid, is negligible.

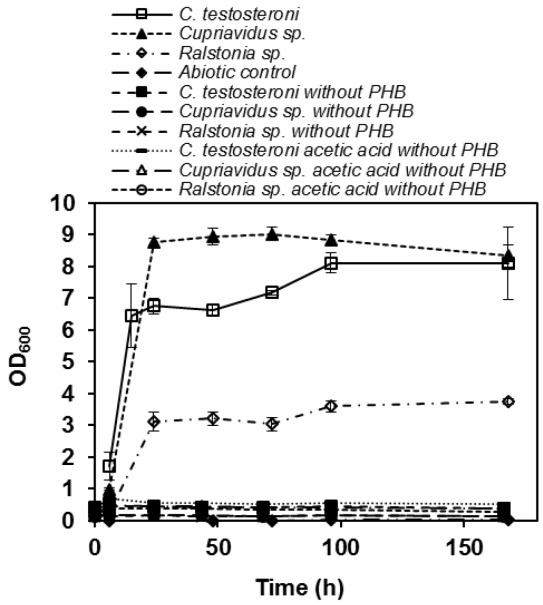

Figure A.1. Additional controls for PHB film exposed to bacterial cultures.
C. testosteroni, Cupriavidus sp.*, Ralstonia* sp., and abiotic control PHB film cultures compared with additional OD_{600} controls without PHB film: 10% v/v inocula with and without 2 µl acetic acid.

A.2. Predicted PhaZs bioinformatics analysis

An additional analysis performed for each of the predicted PhaZs in this study was a protein Basic Local Alignment Search Tool (BLASTp) [2,3] alignment (default conditions) against each of the other predicted sequences, as well as the sequences associated to the demonstrated PhaZs (for *Paucimonas lemoinei* only 5 sequences were selected). Table A.1 shows part of the description tables obtained for significant alignments (Expectation value - E value- less than 10. Lower E values indicate more significant alignments).

It is important to highlight the high similarity between the sequences from *M. algicola* and *P. stutzeri* (70% identities on 94% of the query sequence), which is consistent with the fact that both of these sequences are classified under the homologous family extracellular denatured short chain length PHAs (e-dPHAscl) depolymerases (type 1) homologous family 9 in the PHA Depolymerase Engineering Database (PHA-DED) [4]. The same agreement was seen for the sequences of PhaZ from *L. vestfoldensis* and *Paucimonas lemoignei* PhaZ2 and PhaZ3, which are part of the homologous family e-dPHAscl (type 1) homologous family 11. Then, both sequences from *A. macleodii* and *Comamonas testosteroni* are among the e-dPHAscl (type 2) group, but they belong to different homologous families (7 and 1 respectively). None of the other sequences analyzed (predicted compared to demonstrated) were under the same groups according to the PHA-DED classification, as expected due to lower levels of similarity.

In addition, when the predicted sequences were run against the BLASTp database, high levels of similarities were observed, but mostly with other predicted PhaZs. Among the top 100 alignments, *A. macleodii* had an identities range of 99%–40% (query cover 100%–80%) including predicted PhaZs from other *Alteromonas* species at the top; *L. vestfoldensis* 85%–54% (query cover 100%–95%) with a predicted esterase from *Puniceibacterium antarcticum* (83% identity) at the top; *M. algicola* 98%–25% (query cover 100%–32%) with a domain-containing protein from *Marinobacter* sp. MCTG268 (97% identity) at the top; and *O. indolifex* 87%–46% (query cover 100%–69%) with a predicted esterase from *Sulfitobacter delicates* (87% identity) at the top. Only *M. algicola* displayed alignments with experimentally investigated extracellular PhaZs, like the ones from *Marinobacter* sp. NK-1 (GenBank: BAC15574.1, 99% query cover, 75% identity)

[5,6], *Pseudomonas stutzeri* (GenBank: BAA32541.1, 94% query cover, 70% identity) [7], and from *Pseudomonas mendocina* DSWY0601 (GenBank: AEZ06355.1, 98% query cover, 67% identity) [8].

A further comparison of the investigated sequences was achieved by generating multiple amino acid sequence alignments using Multiple Alignment using Fast Fourier Transform (MAFFT) [9], Clustal Omega [10,11], and by generating graphical representations with Geneious version 11.1.4 [12]. The MAFFT analysis is included in the main body of Chapter 3. Figure A.2 shows an overview of the alignments obtained using Clustal Omega, as well as the identities heat map generated by each alignment, the alignment of the residues neighbouring the main conserved residues of the catalytic domain (Cat) — histidine (H) oxyanion, the serine, aspartic acid and histidine residues of the catalytic triad — and a specific region of the substrate binding domain (SBD) [13]. This method was generally consistent with the MAFFT analysis in the areas of interest and with the percentages of identities between the sequences. The differences observed between some of the alignments result from the differences in the algorithms used for each method, especially in how each program favours generating gaps; MAFFT uses Fast Fourier Transform to generate multiple alignments [9], while Clustal Omega is based on seeded guide trees and profile hidden Markov model techniques [10,11].

In the case of the oxyanion H in Cat, Clustal Omega did not favour a pronounced gap generation as for MAFFT (Figure 3.7) for the sequences of *A. macleodii* and *C. testosteroni*; however, for the sequences from these strains the MAFFT alignment was poor as the aligned residue was not H in both cases. Also, when using Clustal Omega for the sequences of *A. macleodii* and *P. lemoignei* PhaZ7 in the SBD section, rather than

generating a poor alignment like MAFFT, this program ended the sequence earlier. It is interesting to note that the SBD alignment for *P. stutzeri* differs for MAFFT and Clustal Omega (Figure 3.7 and Figure A.2), and each of these different alignments correspond to two alignments reported in literature [13]: reported PhaZ *Pst*I corresponds to the one generated by MAFFT (Figure 3.7), and PhaZ *Pst*II corresponds to the one from Clustal Omega (Figure A.2).

A quick MAFFT alignment of the predicted sequences of this study to the demonstrated intracellular PhaZ (i-PhaZ) from *Rhodospirillum rubrum* [14] (GenBank: ABC22769.1) shows a good general alignment for sequences of *O. indolifex* and *L. vestfoldensis* and identities of 42% and 21% correspondingly to the sequence of *R. rubrum* (Figure A.3). On the other hand, PhaZ sequences from *M. algicola* and *A. macleodii* had poorer alignments and identities of 20% and 10%, respectively. This short exploration towards intracellular PhaZs suggests some of the predicted sequences which were found non-active could be coding for i-PhaZs.

Table A.1. BLASTp alignments between predicted and demonstrated PhaZs.

PhaZ query sequence (GenBank reference)	BLASTp alignment with other predicted and demonstrated PhaZs			
	PhaZ subject sequence (GenBank reference)	Query cover	E value	Identities
Alteromonas macleodii (AFS36858.1)	*Comamonas testosteroni* 31A (AAA87070.1)	84%	1E-60	37%
	Oceanibulbus indolifex Hel45 (EDQ04457.1)	36%	3E-08	31%
	Paucimonas lemoignei PhaZ3 D precursor (AAB48166.1)	61%	3E-07	29%
	Paucimonas lemoignei PhaZ2 B precursor (AAB17150.1)	41%	0.000002	43%
	Paucimonas lemoignei PhaZ4 precursor (AAA65703.1)	37%	0.000009	28%
	Loktanella vestfoldensis (EAQ05513.1)	58%	0.0002	28%
	Marinobacter algicola DG893 (EDM48791.1)	39%	0.0002	39%
	Ralstonia sp. (BAA04986.1)	35%	0.002	44%
	Cupriavidus sp. (AAA21974.1)	35%	0.002	44%
	Pseudomonas stutzeri (BAA32541.1)	42%	0.007	35%
	Paucimonas lemoignei PhaZ5 A precursor (AAA65705.1)	30%	0.009	28%
	Paucimonas lemoignei PhaZ7 precursor (AAK07742.1)	27%	5.5	29%
Loktanella vestfoldensis (EAQ05513.1)	*Paucimonas lemoignei* PhaZ2 B precursor (AAB17150.1)	93%	2E-79	42%
	Paucimonas lemoignei PhaZ3 D precursor (AAB48166.1)	97%	2E-77	42%
	Oceanibulbus indolifex Hel45 (EDQ04457.1)	82%	1E-44	36%
	Marinobacter algicola DG893 (EDM48791.1)	42%	2E-15	33%
	Cupriavidus sp. (AAA21974.1)	91%	2E-14	24%
	Pseudomonas stutzeri (BAA32541.1)	90%	3E-14	28%
	Ralstonia sp. (BAA04986.1)	91%	4E-14	24%
	Paucimonas lemoignei PhaZ5 A precursor (AAA65705.1)	43%	8E-12	31%
	Paucimonas lemoignei PhaZ4 precursor (AAA65703.1)	43%	1E-08	26%
	Alteromonas macleodii (AFS36858.1)	60%	0.0002	28%
	Comamonas testosteroni 31A (AAA87070.1)	35%	0.0008	35%
Marinobacter algicola DG893 (EDM48791.1)	*Pseudomonas stutzeri* (BAA32541.1)	94%	0.0	70%
	Paucimonas lemoignei PhaZ3 D precursor (AAB48166.1)	33%	1E-19	33%
	Oceanibulbus indolifex Hel45 (EDQ04457.1)	51%	9E-19	24%
	Paucimonas lemoignei PhaZ2 B precursor (AAB17150.1)	27%	5E-18	36%
	Cupriavidus sp. (AAA21974.1)	89%	2E-16	23%
	Loktanella vestfoldensis (EAQ05513.1)	22%	4E-15	33%
	Paucimonas lemoignei PhaZ5 A precursor (AAA65705.1)	58%	3E-14	26%
	Paucimonas lemoignei PhaZ4 precursor (AAA65703.1)	35%	3E-13	28%
	Ralstonia sp. (BAA04986.1)	26%	2E-10	31%
	Alteromonas macleodii (AFS36858.1)	22%	0.0004	39%
	Comamonas testosteroni 31A (AAA87070.1)	35%	0.034	44%
Oceanibulbus indolifex Hel45 (EDQ04457.1)	*Loktanella vestfoldensis* (EAQ05513.1)	67%	1E-44	36%
	Paucimonas lemoignei PhaZ3 D precursor (AAB48166.1)	75%	2E-37	32%
	Paucimonas lemoignei PhaZ2 B precursor (AAB17150.1)	66%	1E-34	33%
	Ralstonia sp. (BAA04986.1)	77%	3E-20	28%
	Cupriavidus sp. (AAA21974.1)	64%	2E-19	28%
	Marinobacter algicola DG893 (EDM48791.1)	73%	6E-19	24%
	Paucimonas lemoignei PhaZ4 precursor (AAA65703.1)	72%	1E-17	27%
	Pseudomonas stutzeri (BAA32541.1)	55%	2E-17	29%
	Paucimonas lemoignei PhaZ5 A precursor (AAA65705.1)	71%	3E-17	25%
	Comamonas testosteroni 31A (AAA87070.1)	44%	6E-09	33%
	Alteromonas macleodii (AFS36858.1)	33%	2E-08	31%

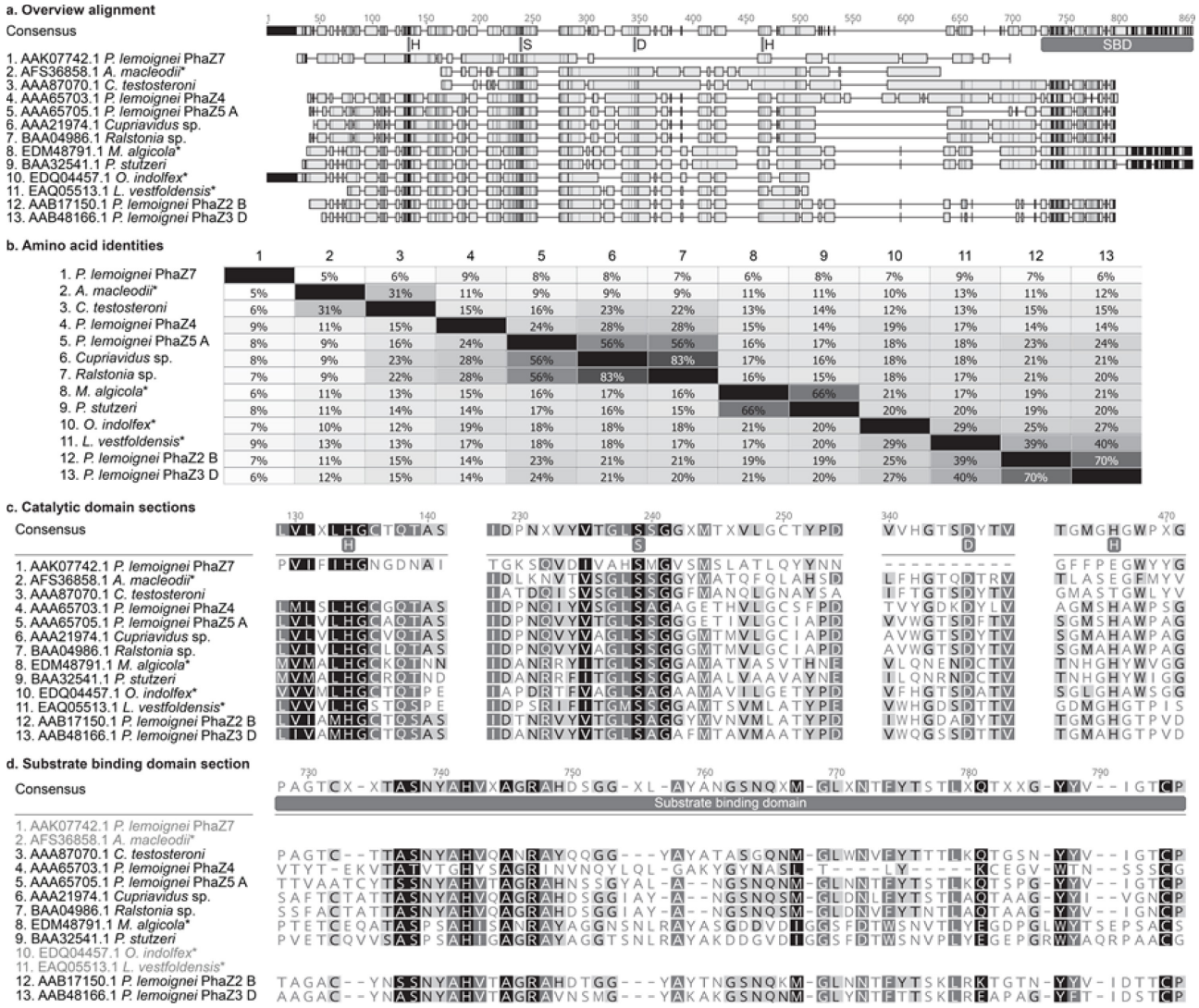

Figure A.2. Comparison of the amino acid sequences of demonstrated and predicted (*) PhaZs by Clustal Omega alignment. (a) Global alignment overview, (b) identities heat map, (c) main sections of the catalytic domain, and (d) part of the estimated location of the substrate binding domain (SBD). Identities are indicated by darker shaded amino acids. Annotations are located below the consensus sequence: oxyanion histidine (first H), catalytic triad amino acids (serine (S), aspartatic acid (D), and second H), and part of the SBD.

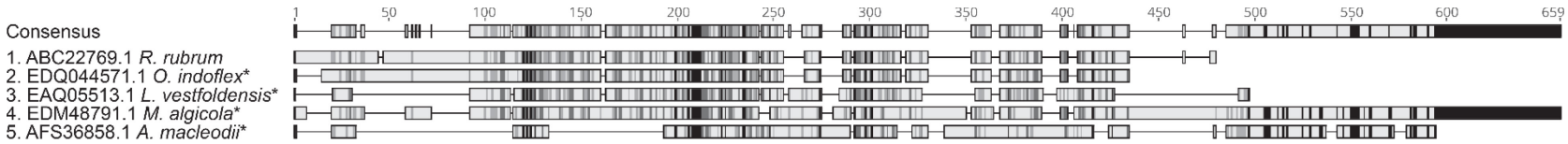

Figure A.3. Global alignment overview of intracellular PhaZ from *R. rubrum* and predicted (*) PhaZs using MAFFT alignment.

A.3. Comparison of PhaZ nucleotide and protein sequences from *Marinobacter* sp. NK-1 and *M. algicola* DG893

Basic Local Alignment Search Tool (BLAST) [2] from the National Center for Biotechnology Information (NCBI) website was used to compare the gene and protein sequences of the PhaZ from *Marinobacter* sp. NK-1 [5,6], and the predicted PhaZ from *M. algicola* DG893. A discontiguous megablast nucleotide alignment between the *Marinobacter* sp. NK-1 *phaZ* gene sequence (GenBank: AB079799.1) and *M. algicola*'s predicted *phaZ* section (GenBank: ABCP01000004.1, REGION: 46935 – 48677) showed 73% identity (1214/1656) between positions 1042 and 2680 for *Marinobacter* sp. NK-1 and positions 48585 and 46941 for *M. algicola*, covering most of the mature peptide nucleotide sequence (Figure A.4). A BLASTp alignment (between GenBank: EDM48791.1 for *M. algicola* and GenBank: BAC15574.1 for *Marinobacter* sp. NK-1) showed 75% identity and 85% positives (taking into account similar residues) between the amino acid sequences (Figure A.5).

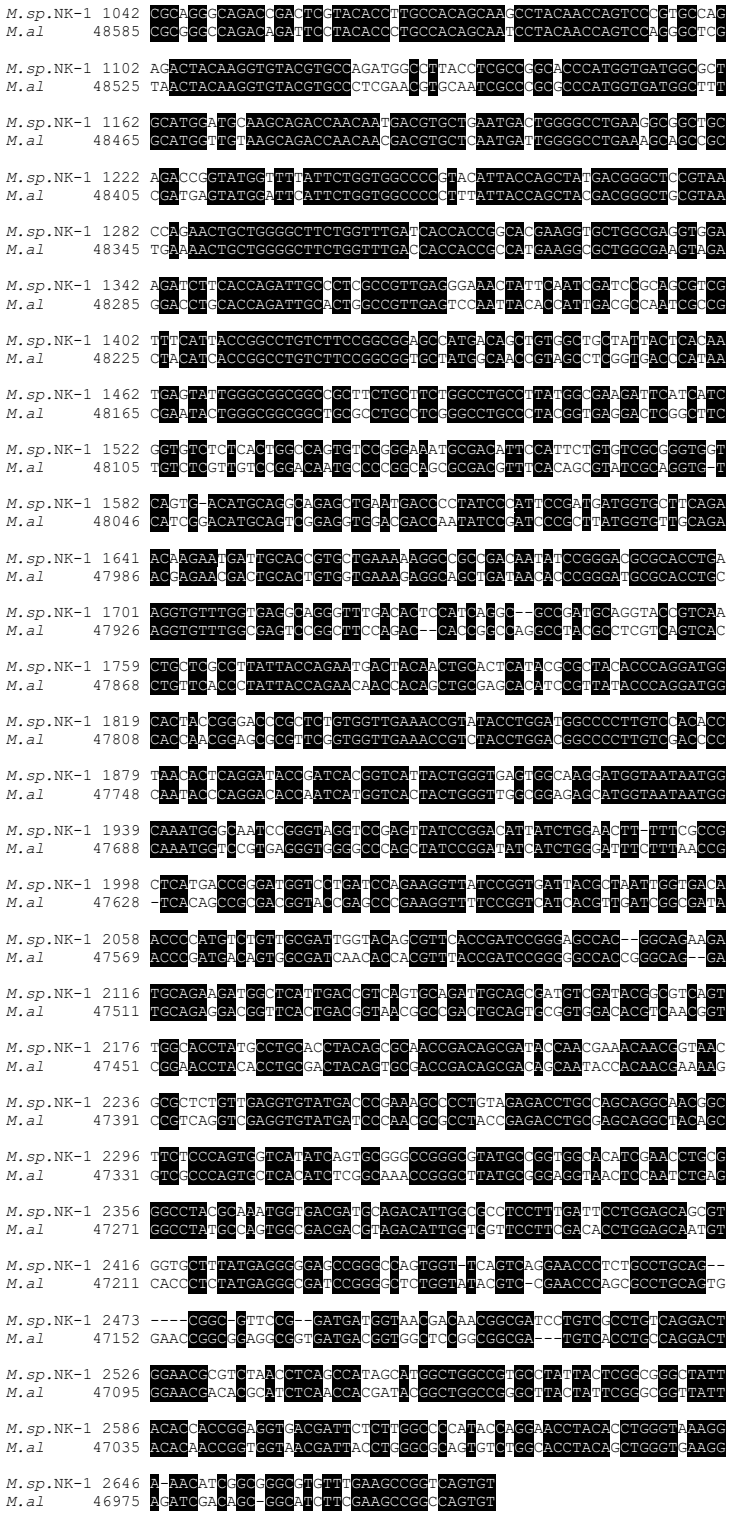

Figure A.4. *Marinobacter* sp. NK-1 and *M. algicola* DG893 *phaZ* nucleotides BLAST. *phaZ* genes: *Marinobacter* sp. NK-1 (upper line) and predicted *M. algicola* DG893 (lower). Homologous regions are shaded and correspond to 73% identity between the genes.

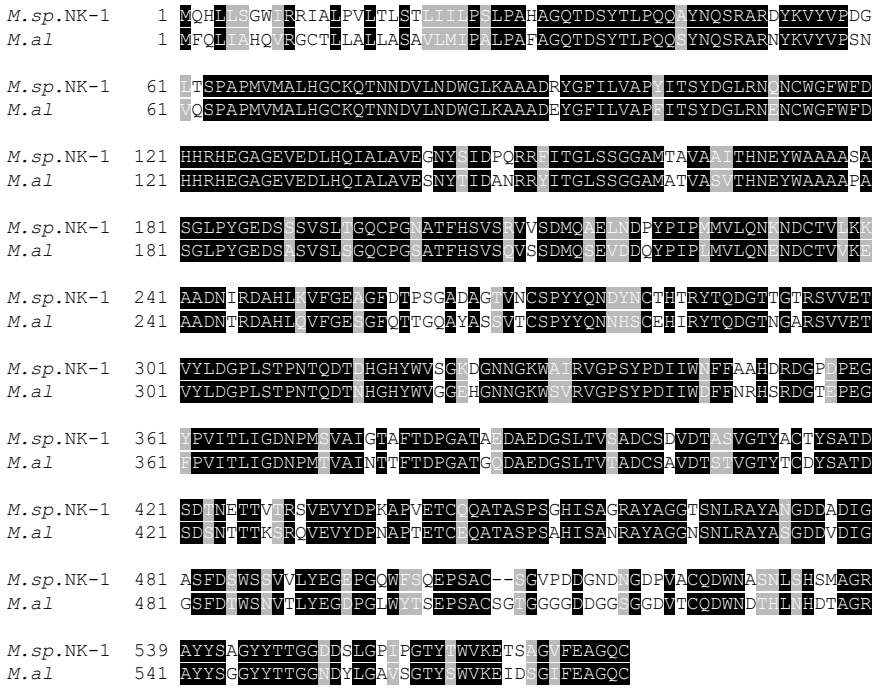

Figure A.5. *Marinobacter* sp. NK-1 and *M. algicola* DG893 PhaZ amino acids BLASTp.

BLASTp between the amino acid sequences corresponding to PhaZ from *Marinobacter* sp. NK-1 (upper line) and the predicted PhaZ from *M. algicola* DG893 (lower). Dark shaded residues indicate homologous regions and correspond to 75% identity. Gray shaded amino acids indicate very similar residues and taken into account**,** the analysis throws 85% positives.

A.4. Polymerase chain reaction (PCR) for confirmation of PhaZs sizes for *Cupriavidus* sp. and *Ralstonia* sp.

In an attempt to clarify and compile information available in the literature on the PhaZs produced by the strains with demonstrated PhaZ activity, we compiled a table (Table 3.2) with protein sizes, sequences references, and other information. Determining the sizes of some of the enzymes from published data proved to be challenging. Particularly, the taxonomy of the organisms studied has changed numerous times over the years and was

not consistent between suppliers, publications and database entries. For instance, until December 2015 and January 2016, *Cupriavidus* sp. and *Ralstonia* sp. were respectively referred to as *Alcaligenes faecalis* and *Ralstonia pickettii* in the Japan Collection of Microorganisms (JCM accession numbers 10169 and 10171). Also, two publications focusing on PhaZ from *Ralstonia pickettii* TI mentioned that this strain was "formally known as *Alcaligenes faecalis*" [15,16]. This conflict was also observed for the GenBank accession number J04223.2 reported by Saito et al. [17], where it is mentioned that the source organism is *Ralstonia pickettii* while the reference publication states the gene belongs to *Alcaligenes faecalis* T1. Additional investigation into the sequence of *Ralstonia* sp. resulted in a PhaZ protein sequence for strain K1, as reported in the accession number D25315.1 (for which its corresponding protein id BAA04986.1 also has 98% identity with the sequence of another protein reported for the strain A1: BAA82057.1), for a mature enzyme size of 47.6 kDa.

Furthermore, it is reported that, in addition to its PHB depolymerase, *Cupriavidus* sp. produces an oligomer hydrolase with a size of approximately 74 kDa (see oligomer hydrolase under GenBank: J04223.2) [18]. Since this particular PhaZ hydrolyses the polymer chains into dimers which are then broken down to monomers by the oligomer hydrolase [18-20], additional monomer units are produced when the strain or extracellular fraction are utilized without prior purification of the PHB depolymerase. For *R. pickettii* A1, an oligomer hydrolase with size of approximately 70 kDa was also reported under the accession number D85373.1 [21]. We were interested to test if the strain used in this study, *Ralstonia* sp., also produced an oligomer hydrolase.

Genomic DNA (gDNA) from *Cupriavidus* sp. and *Ralstonia* sp. was isolated with GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Primers (Table A.2) that contain the restriction sites *Eco*RI and *Xho*I (*Eco*RI for uneven number primers and *Xho*I for even number primers; the restriction sites were added for another study) were designed based on the DNA sequences of PhaZ precursors under GenBank accession numbers J04223.2 (primers 1 and 2), D25315.1 belonging to *R. pickettii* strain K1 — whose *phaZ* sequence is 99% identical to strain A1 accession number AB022287.1 — (primers 3 and 4), and D85373.1 for the oligomer hydrolase from *R. pickettii* A1 (primers 5 and 6). PCR amplification was done with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) in a T100 Thermal Cycler (Bio-Rad). The conditions for PCR were: initial denaturation at 98 °C for 30 s (1 cycle), denaturation annealing at 98 °C for 10 s and extension at 72 °C for 44 s (35 cycles), and final extension at 72 °C for 10 min. The samples were run on a 1% agarose gel at 80 V for 1 h in order to analyze the PCR products.

Table A.2. Primers for potential PhaZ precursors and oligomer hydrolases of *Cupriavidus* sp. and *Ralstonia* sp.

Primer number	Primer sequence (5' to 3')	GenBank accession number
1	CAATTTACCTCTGAATTCGGCCACGGCGGGGCCCGGTGCCT	J04223.2
2	GCGATAAATGCTCTCGAGTGGACAATTGCCGACGATGTAGTAGCCGGCGGCCGTCT	J04223.2
3	CAATTTACCTCGAATTCAGCGGTCACCGCCGGGCCCGG	D25315.1 and AB022287.1
4	GCGATAAATACACTCGAGCGGGCAGTTGCCGATGACGTAGTAGCCGGC	D25315.1 and AB022287.1
5	CCTTTTAGCAGGAATTCAGCATCCGGAGCGATTGGTCTGGCAGGTT	D85373.1
6	GCGATGCGTTGACTCGAGGTCGGCTACTGCGGGCGTATTCGTGAAGTT	D85373.1

Primers 1–2 (Table A.2) designed for the PhaZ precursor under the GenBank accession number J04223.2 were utilized with gDNA from *Cupriavidus* sp. and *Ralstonia* sp. Figure A.6 shows that the primers only worked with the gDNA from *Cupriavidus* sp. with a resulting gene fragment below the 1500-bp marker, which is consistent with the expected size of the sequence of interest, 1420 bp (1383 from the gene plus additional base pairs from the primers). Primer pairs 3–4 and 5–6 were used with the gDNA from *Ralstonia* sp. and both amplifications produced fragments of sizes around 1400 and 2000 bp, respectively.

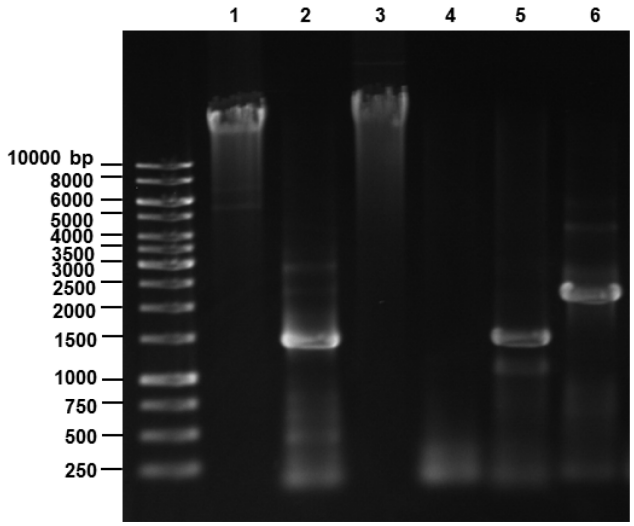

Figure A.6. PCR products using primers for the PhaZ precursor and oligomer hydrolase of *Cupriavidus* sp. and *Ralstonia* sp.
Lanes 1 and 3 were loaded with gDNA of *Cupriavidus* sp. and *Ralstonia* sp., respectively. Lanes 2 and 4 were loaded with amplification products with primer pair 1–2 of gDNA from *Cupriavidus* sp. and *Ralstonia* sp., respectively. Lanes 5 and 6 contain the PCR products of primer pairs 3–4 and 5–6 with gDNA from *Ralstonia* sp.

Analysis of PCR products (Figure A.6) confirmed that the expected size of PhaZ from *Cupriavidus* sp. would be approximately 46.9 kDa based on the corresponding gene

and protein sequences reported in Genbank J04223.2 (Saito et al. 1989). For *Ralstonia* sp., primer pairs 1–2 did not amplify, while the resulting sizes of 1422 and 2085 bp from amplification with primer pairs 3–4 and 5–6, respectively, suggest that this strain produces a PhaZ of similar size as the one reported for strains *R. pickettii* K1 (Genbank D25315.1) [22] and A1 (Genbank AB022287.1), and an oligomer hydrolase of size similar to the one reported for strain *R. pickettii* A1 (Genbank D85373.1) [21] (confirmed by partial sequencing of both *Ralstonia* sp. genes — data not shown). H-NMR analysis performed by Mukai et al. [23] has shown that both monomers and dimers resulted from PHB degradation from *Cupriavidus* sp., while monomers were almost exclusively observed for *Ralstonia* sp. Therefore, since PhaZ from *Ralstonia* sp. hydrolyses the polymer into monomer units efficiently [19], it is unclear what is the role of the oligomer hydrolase in this case; while for *Cupriavidus* sp. its presence would aid in turning the dimeric units produced by PhaZ into monomers to be assimilated by the bacteria [19]. Confirmatory studies on the degradation products in the extracellular fractions and of PHB exposed to recombinant versions of the enzymes produced by these two strains could help clarify this aspect. On the other hand, only one PhaZ has been reported for *C. testosteroni*, and although the name of the strain was changed from *Comamonas* sp. to *Comamonas testosteroni* (German Collection of Microorganisms and Cell Cultures, DSMZ), the information regarding gene and protein sequences is clearer than for *Cupriavidus* sp. and *Ralstonia* sp [24]. In addition, only one PhaZ has been reported for *P. stutzeri* [7], while *P. lemoignei* has seven different PhaZs [13].

A.5. References

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APPENDIX B: Supplementary Material for Chapter 5

B.1. Detection of degradation products by HPLC

Figure B.1 shows HPLC chromatograms of the same samples for which UPLC is shown in Figure 5.6. UPLC results reflected the peaks detected using the initial HPLC analysis.

A slight variation in the detection times was observed in HPLC; this was due to the addition of 600 µl acetonitrile to reach a higher sample volume (a sample without addition of acetonitrile showed lower detection times, data not shown), and possibly to variability during sample processing or sample runs. However, the distance between components remained constant across all samples, allowing correlation between components.

Figure B.1a shows the chromatogram obtained from the liquid fraction of PHB film incubated in mineral medium with 4% NaCl for 30 h at 37 °C (negative control). The BPB peak is seen at approximately 19 min, while the small peak at 17.6 min is associated to a medium component; this result was consistent with negative controls of PHB film incubated with this buffer during 48 h and medium alone (data not shown). Figure B.1b shows the chromatogram for 3HB standard (91.5% purity) at 50 mM, for which the first major peak at 16.2 min is 3HB monomer (confirmed by proportional increases of the first peak and decrease of the BPB peak with additional concentrations of the standard — data not shown), followed by a lower peak at 18.2 min (corresponding to the dimer) and the final main peak corresponding to BPB observed at 19.5 min. For PHB film that reached 2.4% mass loss after incubation with PhaZ$_{Mal}$ (Figure B.1c), the BPB peak appeared at 19.7

min, 3HB monomer at 16.1 min (in agreement to the distance observed with throughout different concentrations of standard), and two smaller peaks close to each other at 18.2 (component in buffer) and 18.4 min (dimer). For PHB film that reached 2.2% mass loss after incubation with PhaZ$_{Cte}$ (Figure B.1d), the BPB peak appeared at 20 min, 3HB monomer at 16.4 min (again, in agreement with the distance between peaks observed throughout different concentrations of standard), and a smaller unknown peak at 18.6 (component in buffer). Higher degradation samples (approximately 11.5%) are shown for PhaZ$_{Mal}$ and PhaZ$_{Cte}$, in Figure B.1e and f, respectively. These show monomer at 16.5 and 16.3 min, no presence of BPB, and peaks at 18.8 and 18.6 min (dimer), respectively for each treatment.

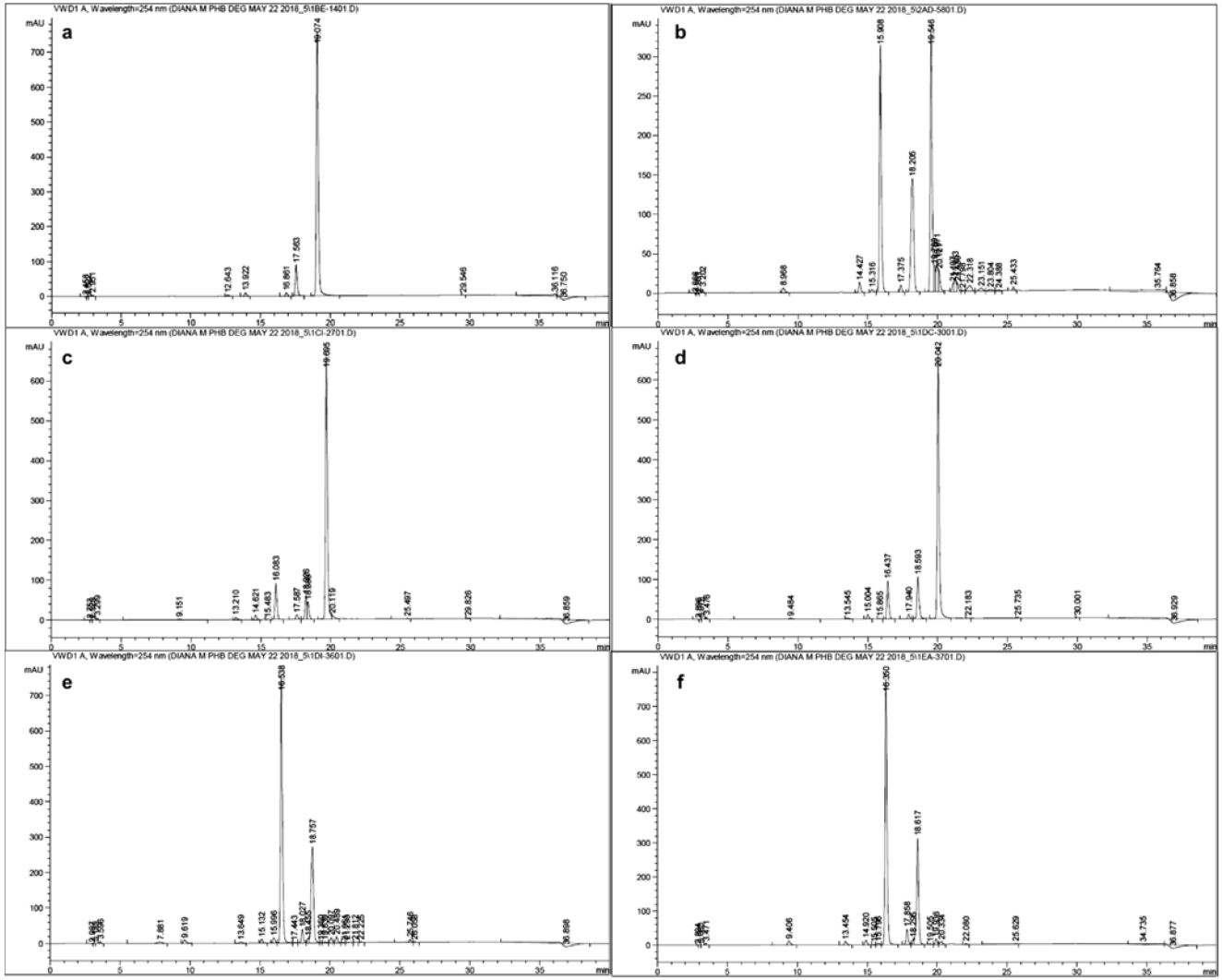

Figure B.1. HPLC of BPB derivates detected at 254 nm.

a) Negative control: Liquid fraction of PHB film incubated in mineral medium with 4% NaCl for 30 h at 37 °C. (b) 50 mM 3HB 91.5% purity. (c) and (d) Liquid fractions of PHB film incubated with PhaZ$_{Mal}$ and PhaZ$_{Cte}$, respectively, in mineral medium with 4% NaCl to a mass loss of ~2%. (e) and (f) Liquid fractions of PHB film incubated with PhaZ$_{Mal}$ and PhaZ$_{Cte}$, respectively, in mineral medium with 4% NaCl to a mass loss of ~12%.

B.2. Additional SEM micrographs

Figure B.2 shows SEM micrographs of both sides of PHB film exposed to PhaZ$_{Mal}$ and PhaZ$_{Cte}$ at approximately 0.5% mass loss. During solvent casting on glass slides, the film surface in contact with air (side 1) showed more pores (more noticeable at 500× magnification), while the side in direct contact with the glass (side 2) was smoother (Figure 5.5).

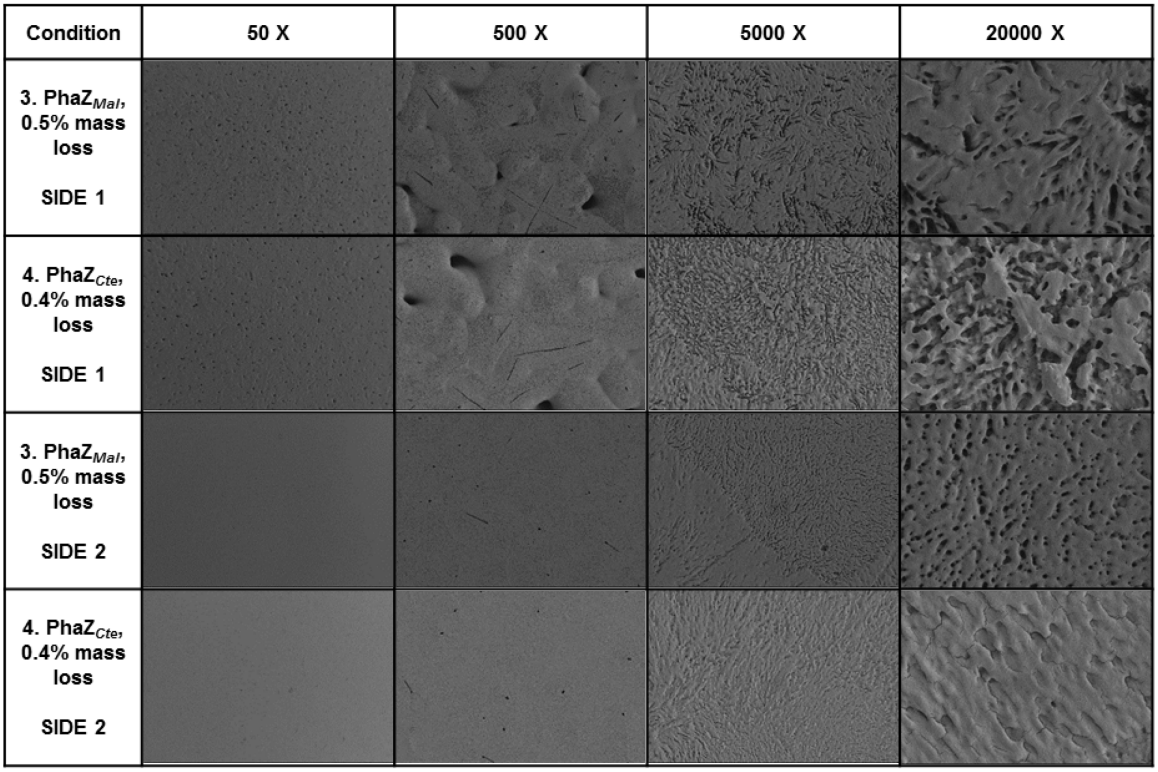

Figure B.2. SEM micrographs of both sides of PHB film exposed to PhaZ$_{Mal}$ and PhaZ$_{Cte}$ at approximately 0.5% mass loss.

APPENDIX C: Supplementary Material for Chapter 6

C.1. Sequencing primers

Table C.1 shows the primers of the primers used for verification of sequences. Primers 1–3 were used to verify the wild-type gene segment of mature PhaZ$_{Cte}$ in PCR products, amplified from gDNA extracted from four *C. testosteroni* colonies. Primers 2–4 were used to verify the CLB variant construct. All other variants were verified with the primers used for pET-22b(+) incorporation listed in Table 6.3.

Table C.1. Sequencing primers used in this study

Primer number	5' to 3' sequence	Description
1	CGGCGGAATTCGATGCGAGTTCAATCCTGGAGAAGC	PhaZ$_{Cte}$ start primer (includes signal peptide sequence) - mature PhaZ$_{Cte}$ verification
2	CTATATCTTCACGGGCACCA	Middle primer - mature PhaZ$_{Cte}$ verification, CLB construct check
3	CCATCAAGGCCATGATCGAT	PhaZ$_{Cte}$ middle primer - mature PhaZ$_{Cte}$ verification, CLB construct check
4	TAATACGACTCACTATAGGG	T7 promoter - CLB construct check

C.2. Variants concentration for PhaZ activity assays

Several concentrations of PhaZ$_{Cte}$ (variant CLB) were tested for PHB depolymerase activity with PHB film using the methods described in Chapter 6, section 6.3.5 (UV-spectrophotometry and mass loss). The results are reported in Figure C.1. The trend for both curves (rate per CLB concentration (ΔOD_{210}.ml)/(µg CLB.s) and mass loss per surface area of film per CLB concentration after 3 h (mg PHB.ml)/(µg CLB.cm^2)) increased from

the lowest concentration (0.25 µg/ml), reaching a maximum at 4 µg/ml for monomer release rate and 2 µg/ml for mass loss data, but both responses are very close for both concentrations. At 8 µg/ml there is a sharp decrease in activity for the monomer release rate and a slight decrease for mass loss. The concentration of 1 µg/ml was selected to perform the variants assays because it showed good measurable activity without reaching a saturation point of enzyme/substrate, which occurred around 2 µg/ml. At this concentration, PhaZ inhibition at high concentrations [1,2] is avoided.

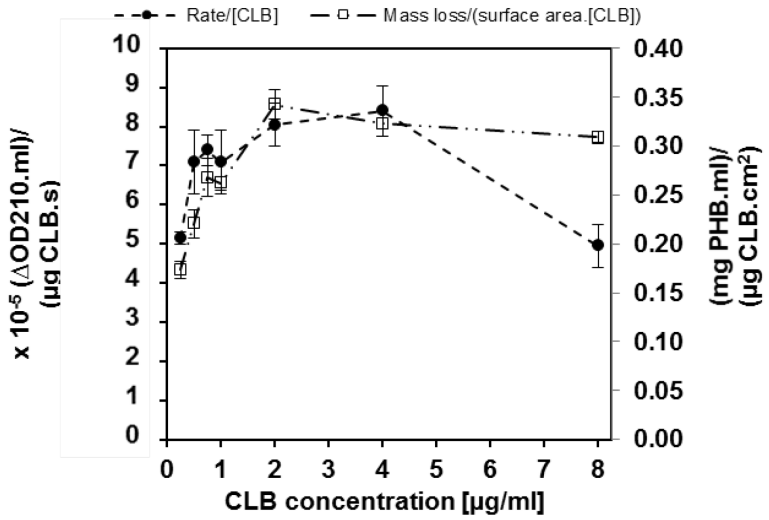

Figure C.1. Activity of CLB variant at different concentrations.
Measurements were made in terms of rate per CLB concentratio [(ΔOD_{210}.ml)/(µg CLB.s)] — due to the production of the monomer 3HB as a product of PHB film hydrolysis during the entire time range (180 min) — and mass loss per surface area of film per CLB concentration after 3 h [(mg PHB.ml)/(µg CLB.cm^2)].

C.3. Statistical analysis additional tests

Normality and homoscedasticity were tested with Shapiro-Wilk (for normality, cannot be tested for n < 3) and Brown-Forsythe (for equal variances) for all groups

analyzed with one-way analysis of variance (ANOVA) — activity data of the PhaZ$_{Cte}$ variants (from UV- spectrophotometry and mass loss), and each variant across storage conditions. The groups that did not meet the normal distribution and equal variance conditions are reported:

For the PhaZ$_{Cte}$ variants comparison (fresh variant), Shapiro-Wilk normality test was not passed for CLS rate $p = 0.0303$, CL mass $p = 0.0239$, and negative control mass loss $p = 0.0012$; Brown-Forsythe test for equal variances reported the standard deviations (SDs) of the rate as significantly different $p = 0.0170$.

For the comparisons of each variant across storage conditions, Shapiro-Wilk normality could not be tested for CLB rate 5 weeks RT and was not passed for SCLB rate 5 weeks 37 °C $p = 0.0282$, and SCLB mass loss 5 weeks RT $p = 0.0361$.

During fresh variants comparison, variant CLB ($1.06 \times 10^{-4} \pm 1 \times 10^{-5}$ (ΔOD_{210}.ml)/(µg variant.s)) compared to SCLB ($8.64 \times 10^{-5} \pm 2 \times 10^{-5}$ (ΔOD_{210}.ml)/(µg variant.s)) were not statistically different for Tukey's multiple comparison for the rate data between 2000 and 4000 s. The slopes were analyzed for the 180 min time range, resulting in $9.37 \times 10^{-5} \pm 3 \times 10^{-6}$ (ΔOD_{210}.ml)/(µg variant.s)) with R^2 0.969 ± 0.01 for CLB, compared to $5.65 \times 10^{-5} \pm 4 \times 10^{-6}$ (ΔOD_{210}.ml)/(µg variant.s)) with R^2 0.997 ± 0.01 for SCLB. These results were found to be statistically different ($t(5) = 13.62$, $p < 0.0001$), which supports the mass loss data comparison.

All analyses were done with GraphPad Prism (version 8.0.0), and the level of significance was set at $p < 0.05$.

C.4. Stability of PhaZ$_{Cte}$ variants – Detailed data

For variants CLB, mCLB, SCLB, and CLS, the results obtained for activity to evaluate stability after different storage conditions (-20 °C, 4 °C, RT, and 37 °C after 1 and 5 weeks) are shown in Figure 6, and in more detail in Table C.2. One-way ANOVA analyses per variant across storage conditions were all positive for statistically different means for rate per variant concentration and mass per surface area of film per variant concentration after 3 h (*p* values in Table C.2, as well as *p* value summary for Dunnett's post-hoc test).

Table C.2. Activity results for PhaZ$_{Cte}$ CLB, mCLB, SCLB, and CLS variants stored at -20 °C, 4 °C, RT, and 37 °C after 1 and 5 weeks.

1–2: linear trend results (2000–4000 s); 3: relative specific activity (2000–4000 s); 4: mass per surface area of film per variant concentration after 3 h. Data is presented as mean ± SD; for 2 and 4 ANOVA *p* values per variant are included and Dunnett's statistical significance summary levels (fresh variant values in Table 5 compared to results under each storage condition, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Storage condition		Result	Variant (one-way ANOVA *p* values: 2. rate, 4. mass loss per surface area at 3 h)			
			CLB ($p = 0.0223$, $p < 0.0001$)	**mCLB ($p < 0.0001$, $p < 0.0001$)**	**SCLB ($p = 0.0236$, $p < 0.0001$)**	**CLS ($p = 0.0016$, $p < 0.0001$)**
1-week	**-20°C**	1. R^2 linear trend	0.998 ± 0.002	0.980 ± 0.02	0.981 ± 0.008	0.995 ± 0.0003
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	9.6 x 10^{-5} ± 3 x 10^{-6}, n=3	1.12 x 10^{-4} ± 5 x 10^{-6}, n=4	8.0 x 10^{-5} ± 2 x 10^{-5}, n=3	3.0 x 10^{-5} ± 1 x 10^{-6}, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	5.0 x 10^{-2} ± 1 x 10^{-3}	5.8 x 10^{-2} ± 3 x 10^{-3}	4.2 x 10^{-2} ± 9 x 10^{-3}	1.55 x 10^{-2} ± 6 x 10^{-4}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.34 ± 0.01, n=2	0.322 ± 0.002, n=4	0.274 ± 0.005, n=3	0.102 ± 0.007, n=4
	4°C	1. R^2 linear trend	0.999 ± 0.0003	1.000 ± 0.0001	0.998 ± 0.003	0.994 ± 0.0004
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	7.4 x 10^{-5} ± 8 x 10^{-6} **, n=3	9.5 x 10^{-5} ± 9 x 10^{-6} *, n=4	7.1 x 10^{-5} ± 1 x 10^{-5}, n=3	2.7 x 10^{-5} ± 3 x 10^{-6}, n=3
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	3.9 x 10^{-2} ± 4 x 10^{-3}	5.0 x 10^{-2} ± 5 x 10^{-3}	3.7 x 10^{-2} ± 6 x 10^{-3}	1.4 x 10^{-2} ± 2 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.49 ± 0.09 ****, n=3	0.30 ± 0.01, n=4	0.29 ± 0.008, n=2	0.12 ± 0.002, n=2
	RT	1. R^2 linear trend	1.000 ± 0.00005	1.000 ± 0.0001	1.000 ± 0.0001	0.996 ± 0.001
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	8.3 x 10^{-5} ± 4 x 10^{-6}, n=3	9.1 x 10^{-5} ± 7 x 10^{-6} **, n=3	8.0 x 10^{-5} ± 6 x 10^{-6}, n=3	2.6 x 10^{-5} ± 3 x 10^{-6}, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	4.4 x 10^{-2} ± 2 x 10^{-3}	4.7 x 10^{-2} ± 4 x 10^{-3}	4.2 x 10^{-2} ± 3 x 10^{-3}	1.4 x 10^{-2} ± 2 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.291 ± 0.006, n=2	0.31 ± 0.01, n=3	0.29 ± 0.02, n=3	0.092 ± 0.008, n=4
	37°C	1. R^2 linear trend	0.998 ± 0.001	0.997 ± 0.005	0.981 ± 0.018	0.997 ± 0.0004
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	9.0 x 10^{-5} ± 3 x 10^{-5}, n=3	1.1 x 10^{-4} ± 1 x 10^{-5}, n=4	9.7 x 10^{-5} ± 1 x 10^{-5}, n=4	2.9 x 10^{-5} ± 2 x 10^{-6}, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	4.7 x 10^{-2} ± 1 x 10^{-2}	5.5 x 10^{-2} ± 5 x 10^{-3}	1.5 x 10^{-2} ± 9 x 10^{-4}	6.7 x 10^{-3} ± 1 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.27 ± 0.01, n=3	0.31 ± 0.02, n=4	0.30 ± 0.01, n=4	0.092 ± 0.004, n=4

Table C.2. (cont.).

Storage condition		Result	Variant (one-way ANOVA p values: 2. rate, 4. mass loss per surface area at 3 h)			
			CLB (p = 0.0223, p < 0.0001)	**mCLB (p < 0.0001, p < 0.0001)**	**SCLB (p = 0.0236, p < 0.0001)**	**CLS (p = 0.0016, p < 0.0001)**
5 weeks	**-20°C**	1. R^2 linear trend	0.999 ± 0.001	1.000 ± 0.0002	0.985 ± 0.010	0.988 ± 0.002
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	1.0 x 10^{-4} ± 1 x 10^{-5}, n=4	1.01 x 10^{-4} ± 7 x 10^{-6}, n=4	8.2 x 10^{-5} ± 1 x 10^{-5}, n=4	2.9 x 10^{-5} ± 2 x 10^{-6}
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	5.2 x 10^{-2} ± 6 x 10^{-3}	5.3 x 10^{-2} ± 4 x 10^{-3}	4.3 x 10^{-2} ± 7 x 10^{-3}	1.5 x 10^{-2} ± 1 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.335 ± 0.006, n=4	0.32 ± 0.01, n=4	0.267 ± 0.008, n=4	0.11 ± 0.01, n=4
	4°C	1. R^2 linear trend	1.000 ± 0.0001	0.999 ± 0.001	0.999 ± 0.0001	0.991 ± 0.003
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	9.1 x 10^{-5} ± 5 x 10^{-6}, n=4	9.7 x 10^{-5} ± 4 x 10^{-6}, n=4	8.2 x 10^{-5} ± 8 x 10^{-6}, n=4	3.0 x 10^{-5} ± 4 x 10^{-6}, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	4.8 x 10^{-2} ± 3 x 10^{-3}	5.1 x 10^{-2} ± 2 x 10^{-3}	4.3 x 10^{-2} ± 4 x 10^{-3}	1.6 x 10^{-2} ± 2 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.29 ± 0.01, n=4	0.29 ± 0.02 **, n=4	0.30 ± 0.02, n=4	0.10 ± 0.01, n=4
	RT	1. R^2 linear trend	1.000 ± 0.0002	1.000 ± 0.0002	1.000 ± 0.0002	0.998 ± 0.0003
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	9.0 x 10^{-5} ± 6 x 10^{-6}, n=3	9.5 x 10^{-5} ± 3 x 10^{-6} *, n=4	9.2 x 10^{-5} ± 4 x 10^{-6}, n=4	3.3 x 10^{-5} ± 3 x 10^{-6}, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	4.7 x 10^{-2} ± 3 x 10^{-3}	5.0 x 10^{-2} ± 1 x 10^{-3}	4.8 x 10^{-2} ± 2 x 10^{-3}	1.7 x 10^{-2} ± 2 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.28 ± 0.01, n=3	0.30 ± 0.01, n=4	0.31 ± 0.02 *, n=4	0.10 ± 0.01, n=4
	37°C	1. R^2 linear trend	0.999 ± 0.0003	0.999 ± 0.0002	0.999 ± 0.001	0.990 ± 0.002
		2. Rate per [variant] [(ΔOD_{210}.ml)/(µg variant.s)]	8.0 x 10^{-5} ± 3 x 10^{-6} *, n=4	8.2 x 10^{-5} ± 3 x 10^{-6} ****, n=4	6.4 x 10^{-5} ± 6 x 10^{-6}, n=4	2.1 x 10^{-5} ± 2 x 10^{-6} **, n=4
		3. Relative specific activity [µg 3HB/(µg variant.s.cm^2)]	4.2 x 10^{-2} ± 2 x 10^{-3}	4.3 x 10^{-2} ± 2 x 10^{-3}	3.3 x 10^{-2} ± 3 x 10^{-3}	1.1 x 10^{-2} ± 1 x 10^{-3}
		4. Mass loss per surface area per [variant] at 3 h [(mg PHB.ml)/(µg variant.cm^2)]	0.249 ± 0.009 **, n=4	0.26 ± 0.01 ****, n=4	0.22 ± 0.01 ***, n=4	0.055 ± 0.003 ***, n=4

C.5. References

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