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This write-up provides a concise yet detailed outline of my doctoral thesis encompassing a few major findings. However, a few pieces of information have been kept confidential and thus are not included in this thesis outline.

This PhD thesis is entitled as “**Marine Biodegradability of Bio-Based Plastics and Chemically Modifiable Polyhydroxyalkanoates**”. This outline provides a brief dissection of the introduction (Chapter 1), 3 major experiments featured in Chapters 2-4, and a summary (Chapter 5).

### **1. Chapter 1: General Introduction**

Plastics, primarily derived from petroleum, are essential to modern life due to their lightweight, durability, transparency, and ease of processing. Their widespread use spans industries such as packaging, electronics, and transportation, with global production reaching approximately 350 million tons annually. However, reliance on non-renewable petroleum resources has raised concerns regarding carbon emissions, resource depletion, and environmental pollution, particularly due to the accumulation of plastic waste in ecosystems. Inefficient recycling and inadequate waste management systems have led to the leakage of 19-23 million tons of plastic waste into aquatic environments each year, harming the marine environment. Unlike natural polymers, petroleum-based plastics resist biodegradation and persist in the environment, breaking down into microplastics that further threaten ecosystems.

To address these challenges, bioplastics, categorized as biodegradable plastics and biomass-based plastics, have emerged as sustainable alternatives. Biodegradable plastics decompose through microbial activity, while biomass-based plastics, derived from renewable sources like sugars and plant oils, reduce dependence on fossil fuels. Among bioplastics, polyhydroxyalkanoates (PHAs) are promising bio-based and biodegradable polymers synthesized by microorganisms as intracellular energy reserves. Since their discovery in 1926,

PHAs have been extensively studied due to their environmental benefits and potential applications in medical and industrial fields. However, the high crystallinity of homopolymers like poly(3-hydroxybutyrate) [P(3HB)] results in brittleness and processing challenges, which researchers aim to overcome by introducing copolymers with longer-chain monomers.

Recent advancements have focused on modifying PHA properties through biosynthetic pathways, particularly by incorporating 2-hydroxyalkanoate (2HA) units. The engineered lactate-polymerizing enzyme (LPE) from *Pseudomonas* sp. has enabled the synthesis of PHA copolymers with enhanced flexibility and processability. Additionally, the functionalization of PHAs through chemical modifications, such as oxidation and polymer side-chain modifications, has led to materials with tunable properties, including hydrophilicity and responsiveness to environmental stimuli.

Biodegradation of plastics involves microbial enzymatic breakdown through four stages: biodeterioration, depolymerization, assimilation, and mineralization. Environmental factors like temperature, oxygen levels, and microbial diversity influence degradation rates, with industrial composting offering the most efficient conditions. While some bioplastics, such as Polylactic acid (PLA), degrade under composting conditions but not in marine environments, PHAs have shown biodegradability in marine systems. Research indicates that polymer crystallinity and molecular weight significantly impact biodegradation rates, with highly crystalline materials exhibiting slower degradation. Studies on P(3HB) depolymerases have revealed specific enzyme-polymer interactions that govern degradation efficiency, highlighting the potential for further material optimization.

This study explores bioplastics for controlled biodegradation and chemical modifications for functional improvements. Chapter 2 examines the biodegradability of synthetic and microbial polyesters in seawater from Suruga Bay, Japan, assessing weight loss, surface morphology, and microbial interactions. Chapter 3 focuses on biosynthesizing P(3HB-*co*-2HA) containing 2-hydroxy-4-methylthiobutyrate (2H4MTB) units using L-methionine as a precursor, followed by oxidative modifications and marine biodegradability assessments. Chapter 4 investigates the biosynthesis of P(3HB-*co*-2HA) containing 2-hydroxy-3-(4-hydroxyphenyl)propionate

(2H3PhOHP) units using L-tyrosine and glucose as precursors, evaluating its material properties and seawater degradation. The findings contribute to developing sustainable plastics that balance performance and environmental responsibility.

### **2. Chapter 2: Biodegradation test of various commercial bio-based plastics in seawater**

Plastics are widely used in daily life, but their accumulation in the environment has become a major concern. Petroleum-derived plastics are highly stable and persist in soil and marine environments. Once released, they undergo weathering and break down into microplastics, posing severe ecological risks. Bio-based plastics have emerged as a potential solution, as some polymers are inherently biodegradable. Biodegradation refers to the microbial decomposition of organic materials into inorganic compounds like carbon dioxide and water. However, the biodegradation rate varies significantly depending on environmental conditions, with marine environments being particularly challenging due to low temperatures and microbial activity. Despite this, 19-23 million tons of plastic waste enter aquatic ecosystems annually, highlighting the urgent need to develop biodegradable polymers suitable for marine environments.

Among biodegradable plastics, poly(L-lactic acid) (PLLA) degrades in compost but not in soil or marine environments. In contrast, synthetic biodegradable polyesters such as poly(butylene succinate-*co*-butylene adipate) (PBSA), poly(butylene adipate-*co*-terephthalate) (PBAT), and polycaprolactone (PCL) exhibit varying levels of biodegradability. Some PHAs, including P(3HB) and poly(3-hydroxybutyrate-*co*-12 mol% 3-hydroxyvalerate) (PHBV12), degrade effectively even in marine environments. Notably, poly(3-hydroxy-2-methylbutyrate) [P(3H2MB)], a type of PHA, has the highest melting temperature among biosynthesized PHAs, making it a promising biodegradable polymer.

This study evaluated the biodegradability of several synthetic and microbial polyesters in seawater from Suruga Bay, Japan. Polymer samples were immersed in seawater, and their weight loss, surface morphology, and microbial population changes were analyzed. The results showed that the biodegradation rate followed this order: PHBV12 > PCL > P(3HB) > PBSA > PBAT > P(3HB-*co*-94 mol% 3H2MB), with PLLA showing no biodegradation. Furthermore,

differences in biodegradation rates were observed between surface and deep seawater, suggesting that microbial activity plays a crucial role. Biochemical Oxygen Demand (BOD) experiments confirmed these findings, indicating that 3HB-based polymers and PCL exhibited the highest biodegradability. However, PCL's low melting temperature and poor mechanical properties limit its applicability. Therefore, 3HB-based polymers were identified as the most suitable base materials for further modification to optimize biodegradation rates for specific applications.

### **3. Chapter 3: Biodegradability of oxidized films of polyhydroxyalkanoate copolymers containing 2-hydroxy-4-methylthiobutyrate unit in seawater**

Extracellular PHA depolymerases play a crucial role in the biodegradation of biopolymers. Many microorganisms secrete these enzymes to hydrolyze biopolymers, utilizing the resulting monomers and oligomers as nutrients, which can ultimately be converted into renewable resources like CO<sub>2</sub> and biomass. The first step in biopolymer recycling is the enzymatic breakdown of polymers into smaller units by PHA depolymerases. Among them, PHB depolymerases are the most widely studied. These enzymes typically consist of a catalytic domain (CD), a substrate-binding domain (SBD), and a linker region. The SBD is thought to play a key role in the adsorption and degradation of PHB through hydrophilic and hydrophobic interactions.

This study hypothesizes that enhancing the hydrophilicity of the PHA surface could modulate and slow its degradation, aiming to minimize environmental pollution by controlling the polymer's lifetime in marine environments. Based on previous research, 3HB-based polymers were selected for investigation. A prior study converted L-methionine into 2H4MTB units as monomers for PHA, leading to increased hydrophilicity after oxidation.

In this study, recombinant *Escherichia coli* DH5 $\alpha$  was used to biosynthesize 2H4MTB-containing P(3HB-co-2HA), which was then formed into films and subjected to oxidation treatment using hydrogen peroxide. The chemical modifications were confirmed by nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR) spectroscopy, and Raman

spectroscopy. Contact angle measurements showed increased surface hydrophilicity after oxidation. The marine biodegradability of both oxidized and non-oxidized films was evaluated through weight loss measurements, microbial flora analysis, and BOD tests using seawater from Suruga Bay. Results showed that biodegradation was more significant in deep seawater than in surface seawater. Surprisingly, oxidized films exhibited higher weight loss than non-oxidized ones in deep seawater, contradicting the initial hypothesis that increased hydrophilicity would slow degradation. Instead, changes in crystallinity appeared to be the dominant factor controlling biodegradation. The BOD test further confirmed that oxidation accelerated the degradation rate. These findings highlight that surface modifications can regulate the biodegradation rate of PHAs, providing valuable insights for designing biodegradable materials with controllable lifetimes.

#### **4. Chapter 4: Biosynthesis and biodegradability of polyhydroxyalkanoate copolymers containing 2-hydroxy-3-(4-hydroxyphenyl)propionate unit**

Surface modification of 3HB-based copolymers is a promising strategy for controlling biodegradation rates. Incorporating different chemically modifiable monomers into these copolymers has gained attention. LPE, a mutated PHA synthase from *Pseudomonas* sp. 61-3, enables the biosynthesis of P(3HB-*co*-2HA) by incorporating various 2HA units, such as 2-hydroxy-4-methylvalerate (2H4MV), 2-hydroxy-3-phenylpropionate (2H3PhP), 2H4MTB, derived from leucine, phenylalanine, and methionine, respectively. Tyrosine, an aromatic amino acid with a phenol group, allows further chemical modifications, such as phosphorylation and sulfonation. However, a 3HB-copolymer incorporating 2H3PhOHP derived from tyrosine, have not been previously reported.

This study aimed to biosynthesize P(3HB-*co*-2H3PhOHP) using tyrosine and glucose as precursors and evaluate its material properties and marine biodegradability. The polymer's structure and composition were confirmed via  $^1\text{H}$  and  $^{13}\text{C}$  NMR, while its phenol groups were chemically modified using phosphate compounds, with verification by  $^{31}\text{P}$  NMR. Additional characterizations included gel permeation chromatography (GPC) for molecular weight

distribution, differential scanning calorimetry (DSC) for thermal properties, and contact angle measurements for hydrophilicity. The biodegradation performance of P(3HB-*co*-2H3PhOHP) films was assessed through weight loss measurements in seawater from Suruga Bay, Japan, along with scanning electron microscopy (SEM) analysis before and after immersion.

This study successfully biosynthesized P(3HB-*co*-2H3PhOHP) and confirmed its chemical modification potential. Thermal analysis revealed that the polymer exhibited a higher glass transition temperature ( $T_g$ ) due to aromatic groups, inhibiting crystallization. Contact angle measurements indicated increased hydrophilicity with 2H3PhOHP incorporation. Biodegradation tests showed that P(3HB-*co*-2H3PhOHP) degraded significantly more slowly in deep seawater than P(3HB). This reduced biodegradation rate was attributed to the phenolic hydroxyl groups in 2H3PhOHP, which increased the  $T_g$ , making the polymer more resistant to degradation in the cold deep-sea environment. Meanwhile, the hydroxyl group in the 2H3PhOHP side chain may influence the adsorption of the depolymerase. These findings provide valuable insights into the precise control of biodegradation rates through polymer structure modification.

## 5. Chapter 5: Conclusion

This study explores the biodegradability of 3HB-based copolymers in marine environments, aiming to control their degradation rates for specific applications. Chapter 2 evaluated the biodegradation of various biopolymers in seawater, revealing that 3HB-based polymers exhibited the highest biodegradability. Chapter 3 focused on oxidizing 2H4MTB-containing P(3HB-*co*-2HA), which increased surface hydrophilicity but suggested that crystallinity, rather than hydrophilicity, plays a key role in biodegradation. Chapter 4 introduced P(3HB-*co*-2H3PhOHP), a novel tyrosine-derived copolymer, demonstrating its high glass transition temperature and reduced biodegradation in deep seawater. This study highlights the potential of structural modifications, such as oxidation and functional group incorporation, to tailor biodegradation rates, contributing to sustainable material development.