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Thesis outline

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This write up is to provide a concise yet detailed outline of my doctoral thesis encompassing a few major findings. However, a few information has been kept confidential thus is not included in this thesis outline.

This PhD thesis is entitled as **"Study on biosynthesis of polyhydroxyalkanoates with diverse structures**". In this outline, a brief dissection on introduction (chapter 1), 3 major experiments featured in chapter 2-4 and summary (chapter 5) is provided.

1. Chapter 1: General Introduction

The proliferation of Petroleum-Based Plastics (PBPs) has been propelled by their convenience, resulting in a significant surge in their manufacturing output. The global production of PBPs has escalated from around 0.5 million tonnes in 1950 to more than 460 million tonnes presently. Projections indicate a continued upward trajectory, with an estimated 1231 million tonnes by 2060. However, the recycling processes for PBPs have not kept pace, leading to substantial environmental accumulation and subsequent ecological problems.The extensive use of PBPs has led to plastic pollution, manifesting in macro plastics (visible debris exceeding 5 mm) and micro plastics (minute fragments smaller than 5 mm). While macro plastics are discernible in landfills, water bodies, and coastal regions, microplastics, initially underestimated, have emerged as a critical concern. Microplastics are readily ingested by marine life, consequently undergoing bioaccumulation as they traverse the food chain, endangering marine and human life.The production of PBPs predominantly relies on non-renewable petroleum resources,

exacerbating climate change and other environmental concerns. The compounded effects of plastic pollution and climate change from PBPs usage constitute a dual peril to the ecosystem. Despite these drawbacks, the convenience and benefits of PBPs often overshadow their drawbacks, rendering a shift away from plastics improbable without a viable and sustainable alternative. One prospective alternative lies in polyhydroxyalkanoates (PHA), a microbial polyester produced using renewable carbon sources. PHA has shown to have an ability to be biodegradable due to their biological origin and can mitigate reliance on finite petroleum resources.

Polyhydroxyalkanoates (PHAs) have garnered substantial attention in both the scientific research community and the industrial sector due to their potential as substitutes for conventional plastics. Despite the progress made in this field, a significant disparity persists between the current stage of development and the overarching objective of achieving a complete transition from petroleum-based plastics to PHAs. Ongoing research endeavors are dedicated to addressing this disparity. Within the domain of PHA research, two prominent challenges have been identified: material properties and biodegradability. To address the inherent limitations of PHAs in meeting industrial standards, considerable efforts have been focused on the production of copolymers based on P(3HB). However, a more comprehensive exploration of diverse monomers incorporating PHAs is necessary to facilitate large-scale industrial applications and optimize their material properties. Besides, a comprehensive understanding of the biodegradation process of PHAs remains elusive. This includes aspects such as the role of microbial populations, the metabolic pathways involved, degradation rates, and underlying mechanisms. Precise tracking and vigilant monitoring of the fate of PHAs during the biodegradation process are of paramount importance in order to enhance our understanding and ascertain the feasibility of their use as substitutes for existing plastics. This thesis is underpinned by two principal objectives to address the 2 major lackings

described as above. The first objective centers on the synthesis of copolymers rooted in 3-hydroxybutyrate (3HB) by employing PhaCs derived from evolutionary engineering and from other bacteria. The second objective involves the creation of materials aimed at advancing the comprehension of PHA biodegradation processes. The fulfilment of these dual objectives mandates the biosynthesis of PHAs with diverse structures.

2. Chapter 2: Screening of polyhydroxyalkanoate synthase mutants from random mutagenesis library for an improved polymerization of diverse monomer units

Numerous appealing attributes of Polyhydroxyalkanoates (PHA), particularly its inherent biodegradability in both terrestrial and marine ecosystems, have engendered substantial interest in both research and commercial spheres. However, the pronounced crystalline nature, brittleness, and rigidity inherent to Poly(3-hydroxybutyrate) (P(3HB)) have impeded the utilization of PHA as a biomaterial for industrial applications, given that P(3HB) constitutes the primary constituent within the PHA family. Interestingly, the incorporation of P(3HB) with amorphous middle-chain length PHA (mcl-PHA) has demonstrated an ability to address the aforementioned limitations. The monomer 3-hydroxyhexanoate (3HHx) within the mcl-PHA spectrum has garnered significant attention for its ability to enhance the overall material properties of PHA upon co-polymerization with P(3HB). The PHA synthase derived from *Aeromonas caviae* ($PhaC_{Ac}$) exhibits substrate specificity towards 3HB-CoA and 3HHx-CoA. It has been observed to naturally synthesize P(3HB-*co*-3HHx) when cultivated utilizing fatty acids and vegetable oils as carbon sources. Nevertheless, the incorporation of 3HHx into the copolymer by $PhaC_{Ac}$ has proven inadequate for industrial applications, with the 3HHx fraction within P(3HB-*co*-3HHx) consistently hovering only around a mere 4 mol%. However, to position it as a viable substitute for conventional plastics like polypropylene and polyethylene, the desired 3HHx

monomer fraction within the copolymer needs to be within the range from 10-15 mol%. Despite multiple efforts to efficiently yield P(3HB-*co*-3HHx) with a higher 3HHx fraction, this endeavour remains challenging. The strategy of evolutionary engineering applied to $PhaC_{Ac}$ shows an enhancement to the enzyme's polymerization capability towards 3HHx. Prior investigations have introduced 2 beneficial mutation (asparagine 149, aspartate 171, valine 214, and phenylalanine 518) into the native $PhaC_{Ac}$ enzyme, leading to an enhancement in 3HHx polymerization. Intriguingly, a double mutant termed $PhaC_{Ac}$NSDG, encompassing two beneficial mutations (asparagine 149 to serine (N149S) and aspartate 171 to glycine (D171G)), has exhibited superior 3HHx polymerization capabilities compared to the parent enzyme. This discovery has opened up new avenues for research. This chapter's objective is to generate mutants based on the NSDG to explore further enhancements in the 3HHx polymerization capability of this superior enzyme. The introduction of additional mutations within the *$phaC_{Ac}$NSDG* gene, encoding $PhaC_{Ac}$NSDG, was accomplished through error-prone polymerase chain reaction (PCR). The cumulative impact of these mutations on 3HHx polymerization capacity was evaluated using a PHA-deficient mutant of *Ralstonia eutropha* (*R. eutropha* PHB-4). Clones carrying the *phaCAcNSDG* gene (referred to as clones henceforth) were initially screened via a plating method supplemented with Nile red dye. A total of 54 potential clones were selected from the primary screening. Subsequently, the chosen clones underwent secondary screening utilizing gas chromatography (GC) analysis. This led to the identification of two final clones, and the specifics of the mutation points were examined. A total of five mutation points were identified across the clones, resulting in the generation of five triple-point mutants (comprising two NSDG mutations alongside a newly introduced single mutation point). These newly derived triple point mutants have showed superior performance to the existing best double mutant NSDG for the incorporation of several second monomer into the P(3HB). This is one of the key novel point of this thesis.

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3. Chapter 3: Exploring class I polyhydroxyalkanoate synthases with broad substrate specificity for polymerization of structurally diverse monomer units

Polyhydroxyalkanoate (PHA) synthesis heavily relies on the enzymatic activity of PHA synthases (PhaCs). PhaCs characterized by a broad substrate specificity offer substantial advantages in PHA production due to their ability to facilitate the copolymerization of diverse monomers. Notably, the PhaC enzyme from *Aeromonas caviae* ($PhaC_{Ac}$) stands out for its capability to efficiently generate poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from vegetable oils and fatty acids. What sets $PhaC_{Ac}$ apart is its unique capacity to polymerize both 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) monomers, a characteristic that distinguishes it from other Class I PhaCs. This distinct property positions $PhaC_{Ac}$ as a promising biocatalyst for the production of P(3HB-*co*-3HHx) copolymers. The potential of $PhaC_{Ac}$ has been further enhanced through evolutionary engineering, leading to the development of the $PhaC_{Ac}$NSDG mutant variant. This variant has demonstrated an enhanced proficiency in synthesizing P(3HB-*co*-3HHx) copolymers with an elevated fraction of 3HHx compared to the wild-type enzyme. Additionally, it has exhibited the capability to incorporate additional monomers such as 3-hydroxy-4-methylvalerate (3H4MV) and 3-hydroxy-2-methylbutyrate (3H2MB). Moreover, the $PhaC_{Ac}$NSDG mutant produces P(3HB) with a superior molecular weight compared to the wild-type enzyme. These attributes make the $PhaC_{Ac}$NSDG mutant a highly attractive candidate for advancing the utilization of PHA as an industrial biomaterial, thereby positioning it as a promising biocatalyst.Chapter 2 of this thesis described the superiority of the $PhaC_{Ac}$NSDG variant through evolutionary engineering, involving the introduction of an additional mutation into the double mutant enzyme. This innovative step results in the creation of intriguing triple-point mutant enzymes.

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However, the limited availability of naturally occurring PhaCs with broad substrate specificity poses a challenge to the scalable production of desired PHA materials for commercial applications. Therefore, the exploration of novel PhaCs exhibiting inherent broad substrate specificity becomes crucial, enabling the industrial-scale synthesis of PHA copolymers as comprehensive replacements for conventional plastics. Furthermore, the existence of PhaCs capable of producing high molecular weight PHA is pivotal for the practical utility of PHA materials. Notably, both PhaCAc and its NSDG variant display sensitivity to ethanol. Some bacterial strains, including *Escherichia coli*, produce ethanol as a metabolite, which functions as a chain transfer agent during the polymerization process. Consequently, when *E. coli* is utilized as the host organism for production, this sensitivity results in the production of PHA materials with relatively low molecular weight.In the research effort outlined in Chapter 3, the pursuit of naturally occurring novel PhaCs possessing exceptional polymerization capabilities and broad substrate specificity culminated in the identification of four newly discovered PhaC variants. These promising PhaCs were identified through bioinformatics, employing the amino acid sequence of $PhaC_{Ac}$ as a template for Basic Local Alignment Search Tool (BLAST) analysis. These novel PhaCs were sourced from bacterial species including *Ferrimonas marina*, *Plesiomonas shigelloides*, *Shewanella pealeana*, and *Vibrio metschnik*ovii. Subsequently, each of these PhaCs was individually expressed in *E.coli* LSBJ, enabling the synthesis of P(3HB) and the incorporation of diverse monomers such as 3HHx, 3H4MV, 3H2MB, and 3-hydroxypivalate (3HPi) into 3HB-based copolymers. With the exception of PhaC from *S. pealeana,* all the PhaC enzymes identified in this study exhibit the capability to incorporate targeted monomers. Among the recently discovered PhaCs, $PhaC_{Ps}$ originating from *P. shigelloides* exhibit the most promising performance, motivating further endeavors to enhance their attributes via protein engineering. The resultant variant, $PhaC_{Ps}$NG, demonstrates superior efficacy in polymerizing the 3H2MB monomer when compared to both $PhaC_{Ac}$ and its

NSDG variant. Additionally, $PhaC_{Ps}NG$ showcases enhanced P(3HB) synthesis characterized by an ultrahigh molecular weight and a low polydispersity index (PDI). These findings underscore the exceptional potential of these newfound PhaC enzymes, positioning them as versatile catalysts for the large-scale manufacture of 3HB-based copolymers. This study has therefore reported 2 key novel points, which are the newest way of screening potential PhaC with broad substrate specificity by means of bioinformatics and the newly derived intriguing PhaCs from various bacteria.

4. Chapter 4: Biosynthesis of P(3HB) with carbon 13 isotope

Polyhydroxyalkanoates (PHAs) have garnered considerable attention due to their potential applications across diverse industries. Despite ongoing scientific endeavors, a comprehensive understanding of the intricate biodegradation mechanisms of PHAs remains elusive, necessitating further research and knowledge accumulation. To establish PHAs as environmentally friendly alternatives to petroleum-based plastics, a profound comprehension of PHA biodegradation mechanisms is crucial. Carbon-13 labeled PHAs offer a promising avenue for unravelling the complex processes involved in their biodegradation. Accurate tracking of polymer fate during biodegradation can be facilitated through carbon-13 labeling. The PHA structure can be modified to incorporate carbon-13 isotopes, enabling the monitoring of polymer degradation and the fate of its carbon atoms. This labeling technique holds the potential to discriminate PHAs from other carbon sources in the environment, facilitating precise monitoring of PHA degradation products. Examining the distribution of carbon-13 tagged fragments provides vital insights into the extent of PHA degradation and the efficacy of biodegradation processes. Furthermore, polymers labeled with carbon-13 offer the advantage of precise quantification of their breakdown. This enables estimation of polymer degradation rates by tracking the temporal change in carbon-13 labeled PHA content. Such quantitative analysis permits comparisons between diverse

degradation conditions or microbial communities, offering valuable insights into the kinetics of PHA biodegradation. Understanding the rate of PHA degradation is pivotal for assessing the efficiency of biodegradation strategies and optimizing PHA degradation processes, thus advancing its potential as a superior substitute for conventional plastics. Carbon-13 labeled synthetic polymers have proven instrumental in identifying specific microbial biomass and their activity during biodegradation8. Similarly, carbon-13 labeled PHA can aid in gauging microbial activity levels during biodegradation. Monitoring the incorporation of carbon-13 isotopes into microbial biomass facilitates the identification of actively participating microbial populations involved in PHA degradation. This knowledge informs the enhancement of bioprocesses for effective degradation and enhances comprehension of microbial consortia governing PHA biodegradation. Analysing carbon-13-labeled microbial biomass also sheds light on the metabolic pathways employed by microbes in PHA breakdown.While Chapters 2 and 3 focused on synthesizing PHA with diverse monomer units to cater to various applications, this chapter is dedicated to crafting a distinctive PHA structure, poly(3-hydroxybutyrate) [P(3HB)], labeled with carbon-13. This endeavor seeks to bridge the research gap concerning the in-situ biodegradation process of PHA. Thus, the objective of this study is to biosynthesize highly carbon-13 labeled P(3HB), presenting an innovative concept poised to offer multifaceted insights into comprehending P(3HB) biodegradation and expanding the horizons of research in this domain. This study has achieved successful biosynthesis of highly carbon 13-labeled poly(3-hydroxybutyrate) [P(3HB)], representing a significant breakthrough. This finding bears substantial importance as it introduces new avenues for research, augmenting our understanding of the authentic on-site biodegradation trajectory of PHA. Furthermore, the investigation establishes the feasibility of generating carbon 13-labeled PHA under both nutrient-abundant and nutrient-scarce conditions, employing both indigenous and non-indigenous PHA producers. Significantly, the study underscores that heightened labeling ratios are attained under

nutrient-restricted conditions, characterized by stringent and directed carbon flux originating from the supplied carbon 13-labeled glucose source.

5. Chapter 5: Summary

In conclusion, this doctoral study has successfully undertaken the systematic synthesis of a polyhydroxyalkanoates (PHA) with diverse structures. The primary objective has been to enhance the mechanical and material attributes of poly(3-hydroxybutyrate) [P(3HB)], while simultaneously offering insightful revelations into the intricate aspects of its biodegradation. These findings hold the potential to pave novel pathways towards the commercialization of PHA materials.