# IDENTIFICATION AND CHARACTERISATION OF ENOYL-COA HYDRATASES (PhaJ/MaoC) FROM NON-MCL PHA ACCUMULATING BACTERIA FOR THE PRODUCTION OF P(3HB-*co*-3HHX) COPOLYMER IN TRANSFORMANT STRAINS

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**by**

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## TABLE OF CONTENTS

## LIST OF TABLES

**Page**

## LIST OF FIGURES

**Page**

## LIST OF SYMBOLS, UNITS, AND ABBREVIATIONS

| | |
|---|---|
| – | Minus |
| % | Percentage |
| ± | Plus minus |
| × | Times |
| × *g* | Times gravity |
| °C | Degree Celsius |
| β | Beta |
| (*R*) | Rectus isomer |
| (*S*) | Sinister isomer |
| µg | Microgram |
| µL | Microliter |
| µm | Micrometer |
| µM | Micromolar |
| 3H4MV | 3-hydroxy-4-methylvalerate |
| 3HA-CoA | 3-hydroxyacyl-CoA |
| 3HB | 3-hydroxybutyrate |
| 3HB-CoA | 3-hydroxybutyryl-CoA |
| 3HD | 3-hydroxydecanoate |
| 3HHx | 3-hydroxyhexanoate |
| 3HHx-CoA | 3-hydroxyhexanoyl-CoA |
| 3HO | 3-hydroxyoctanoate |
| 3HV | 3-hydroxyvalerate |
| 4HB | 4-hydroxybutyrate |

| | |
|---|---|
| 5HV | 5-hydroxyvalerate |
| $A_{260}/A_{280}$ | Ratio of absorbance at 260 nm and 280 nm |
| ACP | Acyl carrier protein |
| bp | Base pair |
| CDW | Cell dry weight |
| CME | Caprylic methyl ester |
| CoA | Coenzyme A |
| CPKO | Crude palm kernel oil |
| Ct | Threshold cycle |
| D | Aspartic acid |
| Da | Dalton |
| DNA | Deoxyribonucleic acid |
| DSC | Differential scanning calorimetry |
| EDTA | Ethylenediaminetetraacetic acid |
| FabG | 3-ketoacyl-CoA reductase |
| FadA | 3-ketoacyl-CoA thiolase |
| FadB | Enoyl-CoA hydratase |
| FadD | Acyl-CoA synthetase |
| FadE | Acyl-CoA dehydrogenase |
| g | Gram |
| GC | Gas chromatography |
| GPC | Gel permeation chromatography |
| H | Histidine |
| h | Hour |
| Had | 3-hydroxyacyl-CoA dehydrogenase |

| | |
|---|---|
| kb | Kilobase |
| kPa | Kilopascal |
| L | Liter |
| LB | Lysogeny broth |
| M | Molar |
| mA | Milliampere |
| MaoC | Enoyl-CoA hydratase or MaoC-like hydratase |
| MCL | Medium-chain-length |
| mg | Milligram |
| min | Minute |
| mL | Milliliter |
| mM | Millimolar |
| MM | Minimal medium |
| $M_n$ | Number-average molecular weight |
| mol% | Mole percent |
| MPa | Megapascal |
| $M_w$ | Weight-average molecular weight |
| ng | Nanogram |
| nm | Nanometer |
| NR | Nutrient rich |
| OD | Optical density |
| $OD_{600nm}$ | Optical density at wavelength 600 nm |
| PE | Polyethylene |
| PP | Polypropylene |
| PS | Palm stearin |

| | |
|---|---|
| P(3HB) | Poly(3-hydroxybutyrate) |
| P(3HB-*co*-4HB) | Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) |
| P(3HB-*co*-3HHx) | Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) |
| P(3HB-*co*-3HV) | Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) |
| P(3HB-*co*-3HHx-*co*-3HO) | Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) |
| P(3HB-*co*-3HV-*co*-3HHx) | Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) |
| P(3HB-*co*-4HB-*co*-5HV) | Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-5-hydroxyvalerate) |
| PCR | Polymerase chain reaction |
| PDI | Polydispersity index |
| pH | Potential of hydrogen |
| PHA | Polyhydroxyalkanoate |
| PhaA | β-ketothiolase |
| PhaB | Acetoacetyl-CoA reductase |
| PhaC | PHA synthase |
| PhaJ | (*R*)-specific enoyl-CoA hydratase |
| PhaP | Phasin |
| PhaR | Polyhydroxyalkanoate synthesis repressor |
| PKAO | Palm kernel acid oil |
| PO | Palm olein |
| psi | Pounds per square inch |
| PTFE | Polytetrafluorethylene |
| qPCR | Quantitative real-time polymerase chain reaction |
| rpm | Revolutions per minute |

| | |
|---|---|
| s | Second |
| SCL | Short-chain-length |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| sp. | Species |
| TAE | Tris-acetate-EDTA |
| $T_g$ | Glass transition temperature |
| $T_m$ | Melting temperature |
| V | Voltage |
| v/v | Volume per volume |
| $V_{max}$ | Maximum rate of reaction |
| w/v | Weight per volume |
| wt % | Weight percent |

## LIST OF APPENDICES

**PENGECAMAN DAN PENCIRIAN ENOIL-KOA HIDRATASE (PhaJ/MaoC) DARIPADA BAKTERIA BUKAN PENGUMPUL MCL-PHA BAGI PENGHASILAN KOPOLIMER P(3HB-*ko*-3HHX) MENGGUNAKAN BAKTERIA TRANSFORMAN**

## ABSTRAK

Polihidroksialkanoat (PHA) ialah plastik terbiodegradasi yang dihasilkan secara semula jadi oleh mikroorganisma, dimana ia berfungsi sebagai penyimpan karbon dan boleh terbiodegradasi sepenuhnya di alam sekitar. Terdapat dua jenis PHA, iaitu PHA kepanjangan rantai pendek (SCL-PHA), yang monomernya terdiri daripada 3 hingga 5 karbon, dan PHA kepanjangan rantai sederhana (MCL-PHA), yang monomernya terdiri daripada 6 hingga 14 karbon. Kopolimer poli[(*R*)-3-hidroksibutirat-*ko*-(*R*)-3-hidroksiheksanoat] [P(3HB-*ko*-3HHx)], terdiri daripada kedua-dua SCL- dan MCL-PHA, yang mempunyai persamaan rapat dengan plastik konvensional dan menunjukkan potensi yang menjanjikan sebagai pengganti plastik tidak terbiodegradasi. Enoil-KoA hidratase (PhaJ/MaoC) ialah enzim penting dalam kitar oksidasi-β metabolisme asid lemak yang dapat meningkatkan bekalan komposisi monomer 3HHx dalam penghasilan kopolimer. Sifat kopolimer boleh diubah apabila komposisi 3HHx dipelbagaikan. Sehingga kini, enoil-KoA hidratase yang berkesan masih berkurang dan menjadi halangan utama dalam meningkatkan bekalan komposisi monomer 3HHx dalam penghasilan P(3HB-*ko*-3HHx). Justeru, kajian ini bertujuan untuk mengecam dan mencirikan enoil-KoA hidratase yang lebih berkesan untuk mengatasi masalah ini. Dalam kajian ini, analisis bioinformatik telah dijalankan bertujuan untuk mengecam enoil-KoA hidratase daripada bakteria bukan pengumpul MCL-PHA, termasuk *Burkholderia* sp. USM, *Jeongeupia* sp. USM3,

*Aquitalea pelogenes* USM4 dan *Rhodococcus pyridinivorans* BSRT1-1. PhaJ/MaoC telah diklon dalam *Cupriavidus necator* PHB$^{-}$4. Kesemua strain rekombinan yang mempunyai PhaJ/MaoC telah dicirikan dengan biosintesis PHA, berserta analisis ekspresi gen. Kopolimer P(3HB-*ko*-3HHx) telah dicirikan dengan menggunakan analisis GPC dan DSC. Sebanyak 6 gen berbeza yang baru telah dipencilkan daripada bakteria bukan pengumpul MCL-PHA. Strain rekombinan *C. necator* PHB$^{-}$4 yang mengandungi *phaC2*$_{Ra}$ daripada *R. aethirivorans* I24 telah diekspresi bersama dengan gen enoil-KoA hidratase, 6 gen berbeza yang baru dipencilkan dan 3 gen yang pernah dicirikan telah dibina. Semua rekombinan strain telah berjaya mengumpulkan kopolimer P(3HB-*ko*-3HHx) dengan komposisi monomer yang pelbagai, antara 4 hingga 16 mol%. Kandungan PHA yang diperoleh bervariasi dari 43.5 hingga 52.3 wt%, manakala berat sel kering (CDW) berjulat antara 3.4 hingga 4.2 g/L. Enoil-KoA hidratase MaoC1$_{Bs}$ mempunyai keberkesanan yang paling ketara dalam pembekalan monomer 3HHx, menghasilkan penggabungan 3HHx paling banyak dan menunjukkan tahap ekspresi gen tertinggi pada jam ke-12 dan ke-36. Kopolimer telah menunjukkan berat molekul ($M_w$) antara 6.7 hingga 9.0 × $10^5$ Da, serta sifat terma dengan suhu lebur ($T_m$) antara 132 hingga 157°C, yang berpotensi untuk digunakan dalam pelbagai aplikasi. Kesimpulannya, semua enoil-KoA hidratase yang baru dicirikan dalam kajian ini dapat berfungsi dan setiap PhaJ dan MaoC mempunyai keupayaan berbeza yang membenarkan penghasilan kopolimer P(3HB-*ko*-3HHx) dengan komposisi monomer yang diinginkan.

**IDENTIFICATION AND CHARACTERISATION OF ENOYL-COA HYDRATASES (PhaJ/MaoC) FROM NON-MCL PHA ACCUMULATING BACTERIA FOR THE PRODUCTION OF P(3HB-*co*-3HHX) COPOLYMER IN TRANSFORMANT STRAINS**

## ABSTRACT

Polyhydroxyalkanoate (PHA) is a biodegradable plastic produced naturally by microorganisms, where it serves as carbon storage and is completely biodegradable in the natural environment. There are two types of PHA, which are short-chain-length PHA (SCL-PHA), consisting of monomers with 3 to 5 carbons, and medium-chain-length PHA (MCL-PHA), consisting of monomers with 6 to 14 carbons. The poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HHx)] copolymer is composed of both SCL- and MCL-PHA, which has a close resemblance to some conventional plastics and displays promising potential as a substitute for non-biodegradable plastics such as single-use plastic. Enoyl-CoA hydratases (PhaJ/MaoC) are the enzymes essential in the β-oxidation cycle of fatty acid metabolism that can increase the supply of 3HHx monomer compositions in the production of the copolymer. The properties of the copolymer may be altered by varying the 3HHx molar composition. To date, there is a lack of effective enoyl-CoA that can increase the supply of 3HHx monomer to produce P(3HB-*co*-3HHx); therefore, this study aims to identify and characterise more effective enoyl-CoA hydratases to overcome this problem. In this study, the bioinformatic analysis was done to screen for functional PhaJs or MaoCs from non-MCL-PHA accumulating bacteria such as *Burkholderia* sp. USM, *Jeongeupia* sp. USM3, *Aquitalea pelogenes* USM4 and *Rhodococcus pyridinivorans* BSRT1-1. The functional PhaJs/MaoCs

were cloned into *Cupriavidus necator* PHB$^{-}$4. The recombinant strains harbouring the PhaJs/MaoCs were characterised through PHA biosynthesis, and gene expression analysis. The P(3HB-*co*-3HHx) copolymer produced were characterized using GPC and DSC. This study successfully identified 6 new and functional enoyl-CoA hydratases from non-MCL-PHA accumulating bacteria. The recombinant strains of *C. necator* PHB$^{-}$4 harbouring *phaC2*$_{\mathrm{Ra}}$ from *R. aethirivorans* I24 co-expressed with 6 different newly isolated and 3 well-characterised enoyl-CoA hydratases genes were constructed. The recombinant strains successfully accumulated P(3HB-*co*-3HHx) copolymer with diverse 3HHx monomeric compositions, ranging from 4 to 16 mol%. The PHA content obtained varied from 43.5 to 52.3 wt%, whereas the CDW ranged between 3.4 to 4.2 g/L. The enoyl-CoA hydratase of MaoC1$_{Bs}$ showed the highest level of gene expression at both 12 and 36 h, which probably contributed to the highest incorporation of 3HHx. The copolymers produced exhibited weight-average molecular weight ($M_{\mathrm{w}}$) ranging from 6.7 to $9.0 \times 10^{5}$ Da and melting temperature ($T_{\mathrm{m}}$) of 132 to 157°C, which can be applicable in diverse applications. In conclusion, all the newly identified enoyl-CoA hydratases are functional, and each PhaJ and MaoC has a different efficiency that allows for the tailored production of the desired P(3HB-*co*-3HHx).

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Plastics with a wide range of applications have been extensively used and become indispensable to daily life owing to their versatility, durability, and stability. The increased demand for plastic, especially single-use plastics such as containers, grocery bags, and bottles, has contributed significantly to the increase in plastic waste production (Gourmelon, 2015). Both the production of single-use plastics and the generation of plastic waste have increased worldwide throughout the years. However, the primary methods of plastic waste management were just landfill disposal and domestic incineration (Moh et al., 2014; Chen et al., 2021). Plastics have a low biodegradability, and improper waste management of petroleum-based plastic has increased the carbon dioxide emissions that triggered severe environmental problems. To combat the adverse effects of plastics and to meet the global demand for plastics, there is a need for the development of biodegradable polymers with comparable material properties that are more environmentally friendly.

The application of polyhydroxyalkanoate (PHA), which is a biopolyester, can be one of the alternatives to solve these issues. PHA is a type of polyester produced and accumulated in various microorganisms as carbon storage under excess carbon sources but nutrient-limiting conditions (Anderson *et al.*, 1990). The beneficial properties of PHA, including properties similar to commercial plastics and good biodegradability, have made PHA one of the best candidates for replacing traditional petroleum-based plastic in certain applications like single-use plastics and packaging.

PHAs are divided into two main groups, depending on the number of carbon atoms in the monomeric units. The first group is the short-chain-length PHA (SCL-PHA) consisting of 3 to 5 carbon atoms in its monomer units, whereas the second group is the medium-chain-length PHA (MCL-PHA) with 6 to 14 carbons in the monomer units. P(3HB) homopolymer was the first discovered and most well-studied SCL-PHA. Most PHA producers can produce SCL-PHA, for instance, *Cupriavidus necator*, *Burkholderia* sp. USM, *Jeongeupia* sp. USM3, *Aquitalea pelogenes* USM4, *Rhodococcus pyridinivorans* BSRT-1, *Aeromonas caviae*, *Halomonas* spp., and many more (Fukui *et al.*, 1997; Chee *et al.*, 2010a; Ng *et al.*, 2016; Zain *et al.*, 2020; Trakunjae *et al.*, 2021; Park *et al.*, 2024a).

However, P(3HB) homopolymer is a highly crystalline thermoplastic showing stiff and brittle properties that hinder the processibility of P(3HB) and consequently limit their application (Sudesh *et al.*, 2000a; Mifune *et al.*, 2010). Studies have found that the physiochemical and mechanical properties of the homopolymer can be improved by copolymerising 3HB with a second monomer unit forming a combination of SCL-PHA and MCL-PHA (SCL-MCL-PHA) copolymers, for example, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] which are tougher and more flexible than P(3HB) (Doi *et al.*, 1995).

P(3HB-*co*-3HHx) exhibits reduced crystallinity, increased elasticity, as well as it is biodegradable by microorganisms in various environments, including soil and marine (Shimamura *et al.*, 1994; Doi *et al.*, 1995; Wang *et al.*, 2018). Over the past few years, P(3HB-*co*-3HHx) has attracted increasing attention and has been considered for diverse applications such as single-use or disposable items,

packaging, painting, and biomedical applications (Kim *et al.*, 2001; Ebnesajjad, 2012). However, it is necessary to tailor-make P(3HB-*co*-3HHx) with the suitable 3HHx monomer composition to suit its respective applications, as different applications may require different properties.

One of the most significant factors affecting the molar composition of 3HHx is the PHA synthase (PhaC). PhaC is the crucial enzyme for PHA biosynthesis as it determines the type of PHA produced by polymerising various (*R*)-3-hydroxyacyl-CoA substrates into PHA with the release of CoA (Rehm *et al.*, 1999). The incorporation of various types of (*R*)-3-hydroxyacyl-CoA substrates into PHA polymer by PhaC determines the properties of synthesised PHAs, including the molecular weights (Sim *et al.*, 1997; Bhubalan *et al.*, 2011). Therefore, many researchers have devoted their time to finding a good naturally occurring PhaC. Based on the substrate specificity, subunit composition, and amino acid sequence, there are four classes of PhaCs (Steinbüchel *et al.*, 1992; Rehm *et al.*, 1999). PhaC2 from *R. aethirivorans* I24, PhaC2$_{Ra}$ can incorporate P(3HB-*co*-3HHx) with a wide range of 3HHx monomer composition (Budde *et al.*, 2011).

Another factor affecting the molar composition of 3HHx is the metabolic pathways of the strain. Metabolic engineering has been done through genetic modification of microbial strains. This can involve PHA-related and non-PHA-related genes from different metabolic pathways (Fukui *et al.*, 1998b; Reinecke *et al.*, 2009; Mifune *et al.*, 2010; Flores-Sánchez *et al.*, 2020). One of the most well-known proteins affecting the 3HHx monomer composition is enoyl-CoA hydratase (PhaJ) (Fukui *et al.*, 1998b; Tsuge *et al.*, 2000; Tsuge *et al.*, 2003a; Budde *et al.*, 2011). PhaJ is an enzyme that catalyses the addition of a water molecule across the

double bond in enoyl-CoA with *R* stereospecificity, resulting in the formation of (*R*)-3-hydroxyacyl (3HA)-CoA (Eggers *et al.*, 2013; Magdouli *et al.*, 2015).

Based on previous studies, PhaJ had demonstrated an essential role in supplying (*R*)-3HA-CoA of 4 – 6 carbon chain length monomers through intermediates of fatty acid β-oxidation cycle for copolymerisation into P(3HB-*co*-3HHx) (Fukui *et al.*, 1998a). Later, MaoC-like hydratase (MaoC) was also identified with high homology, with PhaJ exhibiting the same functional activity in copolymer production (Park *et al.*, 2003; Wang *et al.*, 2013).

To date, there are just several previous studies focused on isolating and characterising PhaJ enzymes to produce P(3HB-*co*-3HHx) with various 3-hydroxyhexanoate (3HHx) monomer fractions (Fukui *et al.*, 1997; Budde *et al.*, 2011; Insomphun *et al.*, 2015; Tan *et al.*, 2020). Different PhaJs have their respective substrate specificities towards various chain-length monomers. Hence, using different PhaJs allowed the production of P(3HB-*co*-3HHx) copolymers with various 3HHx monomer fraction for various applications. One of the most promising PhaJ that showed excellent efficiency in increasing C6 monomer was PhaJ from *Pseudomonas aeruginosa* (PhaJ1$_{Pa}$) that has high specificity towards C6 and successfully increases the 3HHx fractions up to 78 mol% (Tsuge *et al.*, 2003b).

However, until today, only limited numbers of efficient PhaJs in monomer supply were isolated, and most of the PhaJs were isolated from the MCL-PHA-producing bacteria such as *Pseudomonas* spp. (Tsuge *et al.*, 2003b; Sato *et al.*, 2011; Impallomeni *et al.*, 2018). Therefore, it is necessary to identify more potential *phaJ* genes from various bacteria with different capabilities to control the 3HHx molar fraction to tailor-make the P(3HB-*co*-3HHx) for various applications as it determines the properties of the copolymer produced.

The development of efficient recombinant strains capable of producing P(3HB-*co*-3HHx) copolymer with variable monomer fractions of 3HHx using plant oil, which is crude palm kernel oil (CPKO) as the sole carbon source is essential. It is necessary to attain the desirable P(3HB-*co*-3HHx) copolymer with a desired 3HHx monomer fraction and weight-average molecular weights ($M_w$) as different applications require P(3HB-*co*-3HHx) with distinct 3HHx composition and properties.

In this study, *in-silico* data mining and bioinformatic analysis were done to screen and identify the functional PhaJs and MaoCs from non-MCL PHA accumulating bacteria. Interestingly, non-MCL PHA accumulating bacteria contain genes essential for MCL-PHA production. Subsequently, the ability of putative PhaJs and MaoCs to supply and control the 3HHx monomers were characterized by heterologous co-expression with PhaC2$_{Ra}$. Furthermore, the relationship between the gene expression of *phaJs* or *maoCs* in 3HHx monomer supply was investigated, and the copolymers produced were also characterised.

## 1.2 Problem statement

The most common PHA, P(3HB), is hard, high melting point, and is brittle, which limits its applications. To improve the properties of P(3HB), copolymerisation with 3HHx monomer is necessary. However, only limited strains can produce high yields of P(3HB-*co*-3HHx) with various 3HHx monomers fraction for desirable properties. One common strategy to control the 3HHx monomer fraction in P(3HB-*co*-3HHx) is by overexpressing enoyl-CoA hydratases. However, only a few numbers of effective enoyl-CoA hydratases with the ability to supply 3HHx have been reported to date. Therefore, it is pivotal to identify new functional enoyl-CoA hydratases to produce P(3HB-*co*-3HHx) with a wide range of 3HHx monomer fractions.

## 1.3 Objectives

The main purpose of this research was to identify new *phaJ* or *maoC* genes that are functional and effective in supplying 3HHx monomers for the biosynthesis of P(3HB-*co*-3HHx). The specific research objectives are as follows:

1. To identify and isolate enoyl-CoA hydratases (PhaJ/MaoC) from 4 different non-MCL-PHA accumulating bacteria.
2. To evaluate the efficiency of enoyl-CoA hydratases in increasing 3HHx molar composition in P(3HB-*co*-3HHx).
3. To characterise the P(3HB-*co*-3HHx) produced by *C. necator* recombinant strains.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Polyhydroxyalkanoates (PHAs)

The first type of PHA discovered is P(3HB) which is discovered by Maurice Lemoigne in 1926 in *Bacillus megaterium* (Lemoigne, 1926; Palmeiro-Sánchez *et al.*, 2022). PHAs are polymers of hydroxyalkanoates accumulated intracellularly in discrete inclusions in the cytoplasm as carbon and energy storage compounds by various bacteria when carbon source is abundant, but nutrients such as nitrogen, phosphorus, sulphur, magnesium or oxygen are limited (Anderson *et al.*, 1990; Doi, 1990; Morlino *et al.*, 2023). Over the years, more than a hundred PHA monomer constituents synthesised by different microorganisms have been identified (Rehm, 2003; Wei *et al.*, 2022). PHA accumulation can account for almost 90% of the dried cell weight of bacteria without affecting the intracellular osmotic pressure and does not impair cell function or lead to polymer leakage from the cell (Madison *et al.*, 1999; Verlinden *et al.*, 2007).

One common technique for qualitatively determining PHA-producing bacterial strains is phase contrast microscopy (Dawes *et al.*, 1973; Sudesh *et al.*, 2000a; Salinas *et al.*, 2023). PHA can be observed as light-refracting granules under a phase-contrast light microscope. The size of the PHA granules can vary depending on the organism, ranging from 0.2 to 0.5 μm in diameter, and the largest granule size was recently reported with granule size up to 10 μm (Kim *et al.*, 2001; Shen *et al.*, 2019; Kalia *et al.*, 2024). PHA granules can be explicitly stained with Sudan black or fluorescent dyes such as Nile blue and Nile red (Schlegel *et al.*, 1970; Ostle *et al.*, 1982; Máčalová *et al.*, 2023). In addition, transmission electron microscopy can also

be used as a reliable method for structural analysis of the ultrastructure of PHA granules from thin sections of PHA accumulating bacteria cells (Neoh *et al.*, 2024).

PHAs are attracting worldwide attention and are currently the subject of extensive research because of their physical properties similar to certain petroleum-based synthetic plastics like polypropylene (PP) and polyethylene (PE) and biodegradable at the same time (Lee, 1996; Braunegg *et al.*, 1998; Murugan *et al.*, 2020). Due to the growing interest in PHAs for their attractive physical and material properties and the development of commercial applications, substantial efforts have been made to further develop PHAs for various commercial applications, such as packaging and storage materials.

Figure 2.1 illustrates the general chemical structure of PHAs. The type of PHA can be differentiated based on its side chain or R-group and the number of repeating units of methylene ($CH_2$) group (x) in the chemical structure, as demonstrated in Table 2.1. By altering the functional groups in the side chain of monomers, it is possible to tailor the chemical and physical properties of PHAs produced. The functional groups can be halogen, hydroxyl, phenoxy, epoxy, carboxyl, and cyanophenoxy (Kim *et al.*, 2001; Rai *et al.*, 2011). The advantages of PHAs over petrochemical plastics include their natural origin, renewability, biocompatibility, and susceptibility to degradation by various microorganisms through enzymatic reactions (Mergaert *et al.*, 1993; Sudesh *et al.*, 2000a; Li *et al.*, 2016; Park *et al.*, 2024a).

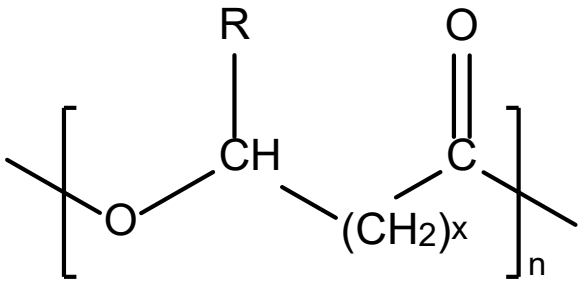


Figure 2.1: The general chemical structure of PHA. R refers to the side chain, x refers to the number of alkane groups on the main chain, and n indicates the number of repeating units. R and x determine the types of hydroxyalkanoate monomer units formed.

Table 2.1: Various hydroxyalkanoate (HA) monomer types with different R and x values (Loo *et al.*, 2007).

| **X** | **R side chain** | **Type of monomer** | **Monomer size** |
|---|---|---|---|
| 1 | Methyl ($CH_3$) | 3-hydroxybutyrate; 3HB | SCL |
| | Ethyl ($C_2H_5$) | 3-hydroxyvalerate; 3HV | SCL |
| | Propyl ($C_3H_7$) | 3-hydroxyhexanoate; 3HHx | MCL |
| | Hydrogen | 3-hydroxypropionate; 3HP | SCL |
| 2 | Hydrogen | 4-hydroxybutyrate; 4HB | SCL |
| 3 | Hydrogen | 5-hydroxyvalerate; 5HV | SCL |

## 2.2 PHA monomer diversity

PHAs are diverse due to the increasing diversities of monomers, homopolymers, copolymer, functional polymers, molecular weights, and combinations of the above. PHA monomers (Table 2.2) can generally be divided into short-chain-length PHAs (SCL-PHAs) and medium-chain-length PHAs (MCL-PHAs) according to the number of carbon atoms its monomer unit possesses. SCL-PHAs have 3 to 5 carbon atoms in the monomer, while MCL-PHAs have 6 to 14 carbon atoms in the monomer.

Table 2.2: Examples of PHAs and PHA producers.

| PHA | PHA monomer | PHA producer | Citation |
|---|---|---|---|
| **SCL-PHA** | 3-hydroxybutyrate (3HB)<br>3 hydroxyvalerate (3HV)<br>3-hydroxypropionate (3HP)<br>4-hydroxybutyrate (4HB) | *C. necator*;<br>*A. pelogenes* USM4;<br>*R. pyridinivorans* B SRT-1; | (Bloembergen *et al.*, 1986; Ng *et al.*, 2016; Trakunjae *et al.*, 2021) |
| **MCL-PHA** | 3-hydroxyhexanoate (3HHx)<br>3-hydroxyoctanoate (3HO)<br>3-hydroxydecanoate (3HD)<br>3-hydroxydodecanoate (3HDD) | *Pseudomonas* spp;<br>*Thermus thermophilus* HB8 | (Huisman *et al.*, 1989; Pantazaki *et al.*, 2009; Wang *et al.*, 2017; Impallomeni *et al.*, 2018) |

## 2.3 Properties of PHA

The monomer composition of PHA can influence the PHA polymer's mechanical, thermal, and physical properties. SCL-PHA typically possesses thermoplastic properties, such as high crystallinity, stiffness, brittle, high melting temperature, and low elongation at break, making them hard to process and not suitable for many applications where the flexibility of the polymer was necessary (Sudesh *et al.*, 2000a).

MCL-PHA has elastomeric properties such as low crystallinity, melting temperature, and high elongation at break (Sudesh *et al.*, 2000a; Kim *et al.*, 2007; Rai *et al.*, 2011). However, MCL-PHA is amorphous, sticky, and viscous at temperatures above or close to the polymer's melting temperature (Hazer *et al.*, 2007; Li *et al.*, 2016). Different functional groups in the side chains of MCL-PHA can alter their physical properties, extending the potential application of the polymer, especially for biomaterial for biomedical applications such as tissue engineering (Zinn *et al.*, 2001; Kim *et al.*, 2007).

PHA could be synthesised as either homopolymer or heteropolymer, where homopolymers contain only one type of PHA monomer, whereas heteropolymers comprise more than one type of PHA monomer. Due to the drawbacks of SCL-PHAs and MCL-PHAs homopolymers, the idea of copolymerisation was introduced, giving rise to the combination of SCL-MCL-PHA copolymers.

The SCL-MCL-PHA shows improved thermal and physical properties for a broader range of applications than homopolymers of SCL- and MCL-PHAs (Noda *et al.*, 2005). PHA copolymers are more flexible and have lower melting temperatures compared to P(3HB) homopolymers, potentially improving their processability and

performance. Examples of copolymers are P(3HB-*co*-3HHx), P(3HB-*co*-4HB) poly(3-hydroxybutyrate-*co*-3-hydroxyoctanoate) [P(3HB-*co*-3HO)], and many more (Rai *et al.*, 2011). There are also studies reported on terpolymers including poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) [P(3HB-*co*-3HHx-*co*-3HO)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HV-*co*-3HHx)], and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-5-hydroxyvalerate), P(3HB-*co*-4HB-*co*-5HV) (Bhubalan *et al.*, 2010; Sun *et al.*, 2010; Lakshmanan *et al.*, 2019).

### 2.3.1 Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), P(3HB-*co*-3HHx)

P(3HB-*co*-3HHx) was reported to have promising characteristics and has caught the tremendous interest of many researchers in exploring and producing this polymer. Figure 2.2 shows the chemical structure of P(3HB-*co*-3HHx). P(3HB-*co*-3HHx) exhibits a lower degree and rate of crystallization as the molar composition of 3HHx increases (Volova *et al.*, 2021). The X-ray crystallinity of the P(3HB-*co*-3HHx) copolymer decreased steeply from 60 to 18% as the 3HHx fraction increased from 0 to 25 mol% (Doi *et al.*, 1995; Tanadchangsaeng *et al.*, 2010). A lower crystallisation rate will result in a more elastic polymer with an elongation at break of 850% when the 3HHx monomer composition was 17 mol%. The melting temperature ($T_m$) of P(3HB) homopolymer is 180°C, and with the increased incorporation of 3HHx monomer compositions, the $T_m$ also decreases. A study by Murugan *et al.* (2017) showed that the $T_m$ of the copolymer decreased from 171 to 156°C when the 3HHx monomer fractions increased from 4 to 15 mol%.

The $M_w$ of the P(3HB-*co*-3HHx) may also be affected by the monomer fractions of the 3HHx and incorporation of 3HHx into the P(3HB) results in

copolymer with lower $M_w$ than the P(3HB) homopolymer. For instance, the $M_w$ for P(3HB-*co*-3HHx) with a range of 3 to 13 mol% of 3HHx, showed a decrease in $M_w$ from $17.3 \times 10^5$ to $8.0 \times 10^5$ Da (Fiorese *et al.*, 2009; Tan *et al.*, 2020). This can be attributed to the nature of the 3HHx monomer, which is bulkier than the 3HB monomer, the decreased PhaC turnover rate, and the increase in the rate of chain transfer reaction (Tomizawa *et al.*, 2010; Wong *et al.*, 2012; Murugan *et al.*, 2017).

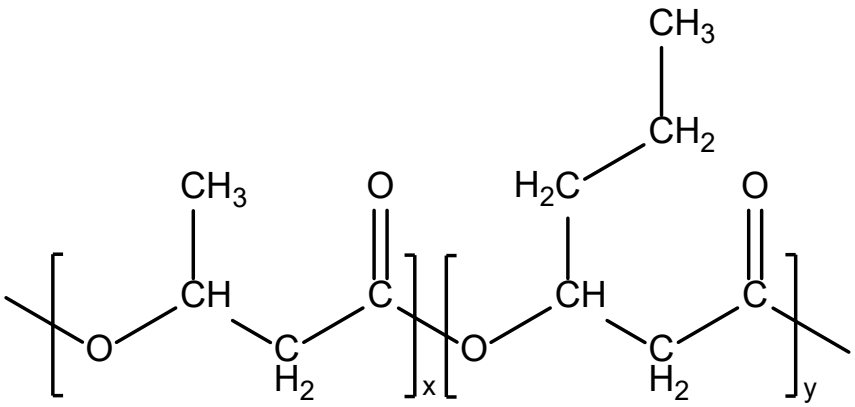


Figure 2.2: Chemical structure of P(3HB-*co*-3HHx). x and y correspond to the number of each repeating unit in the copolymer.

## 2.4 Metabolic pathways of PHA biosynthesis

Several PHA biosynthetic pathways have been identified to synthesise (*R*)-3-hydroxyacyl-CoA, including natural PHA synthesis pathways in organisms and engineered pathways (Meng *et al.*, 2014). Several important metabolic pathways like the tricarboxylic acid cycle (TCA), glycolytic and pentose phosphate pathway, amino acid catabolism, and fatty acid degradation pathways are directly or indirectly linked to the PHA biosynthetic pathways (Madison *et al.*, 1999).

The most common pathway for the PHA biosynthesis pathway is the P(3HB) biosynthesis pathway. In PHA studies, the most common bacteria used is *C. necator*. *C. necator* H16 is a model organism for understanding PHA biosynthesis, as it can accumulate a high amount of P(3HB) (Anderson *et al.*, 1990; Reinecke *et al.*, 2009). The P(3HB) biosynthesis pathway comprises three main enzymatic reactions, each catalysed by distinct enzymes (Madison *et al.*, 1999). The main steps of this pathway are condensation, reduction, and polymerisation. The β-ketothiolase (PhaA) initiates the process by condensing two acetyl-CoA molecules forming acetoacetyl-CoA. Acetoacetyl-CoA is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). PhaC then catalyses the polymerisation of 3-hydroxybutyryl-CoA (3HB-CoA) into P(3HB) (Figure 2.3).

PHA producers can incorporate different PHA monomers from structurally related or non-related carbon sources to produce various PHA monomers other than 3HB. The MCL-PHA monomers can be converted from intermediates of fatty acid β-oxidation and *de novo* synthesis pathway. For example, the formation of 3HHx-CoA through hexenoyl-CoA from β-oxidation using fatty acids or oil as carbon sources (Doi *et al.*, 1995; Fukui *et al.*, 1997).

In the fatty acid β-oxidation cycle, microorganisms metabolise fatty acids and convert them into acyl-CoA using acyl-CoA synthetase (FadD). The acyl-CoA is oxidised into enoyl-CoA by acyl-CoA dehydrogenase (FadE). Enoyl-CoA hydratase then converts enoyl-CoA into (*S*)-3-hydroxyacyl-CoA. Subsequently, the (*S*)-3-hydroxyacyl-CoA is oxidised by 3-hydroxyacyl-CoA dehydrogenase, forming 3-ketoacyl-CoA. 3-ketoacyl-CoA will be converted to acyl-CoA catalysed by 3-ketoacyl-CoA thiolase with the release of one acetyl-CoA. The acetyl-CoA generated by the β-oxidation pathway can also enter the P(3HB) biosynthesis pathway to generate PHA by undergoing similar processes previously described. Besides that, the 3-ketoacyl-CoA reductase (FabG) can also catalyse the conversion of 3-ketoacyl-CoA back into (*R*)-3-hydroxyacyl-CoA, which can be polymerised by PhaC into corresponding monomers. (*R*)-3-hydroxyacyl-CoA hydratase (PhaJ) can also catalyse the conversion of enoyl-CoA into (*R*)-3-hydroxyacyl-CoA, which can be incorporated by PhaC into PHA polymer (Figure 2.3) (Fukui *et al.*, 1997; Kim *et al.*, 2007; Magdouli *et al.*, 2015).

In the fatty acid *de novo* synthesis pathway, malonyl-CoA-ACP transacylase (FabD) will first catalyses the conversion of malonyl-CoA to malonyl-ACP. Subsequently, β-ketoacyl-ACP synthase (FabB) then catalyses the synthesis of 3-ketoacyl-ACP. Finally, 3-ketoacyl-ACP is converted by 3-ketoacyl-ACP reductase (FabG) into (*R*)-3-hydroxyacyl-CoA, readily polymerised by corresponding PhaC. The MCL-PHA monomers could also be channelled from the fatty acid *de novo* biosynthesis pathway, whereby 3-hydroxyacyl-ACP-CoA transferase (PhaG) links the two paths by converting (*R*)-3-hydroxyacyl-ACP to (*R*)-3HA-CoA. The (*R*)-3HA-CoA can then be incorporated into the PHA polymer (Figure 2.3) (Rehm *et al.*, 1998; Philip *et al.*, 2007).

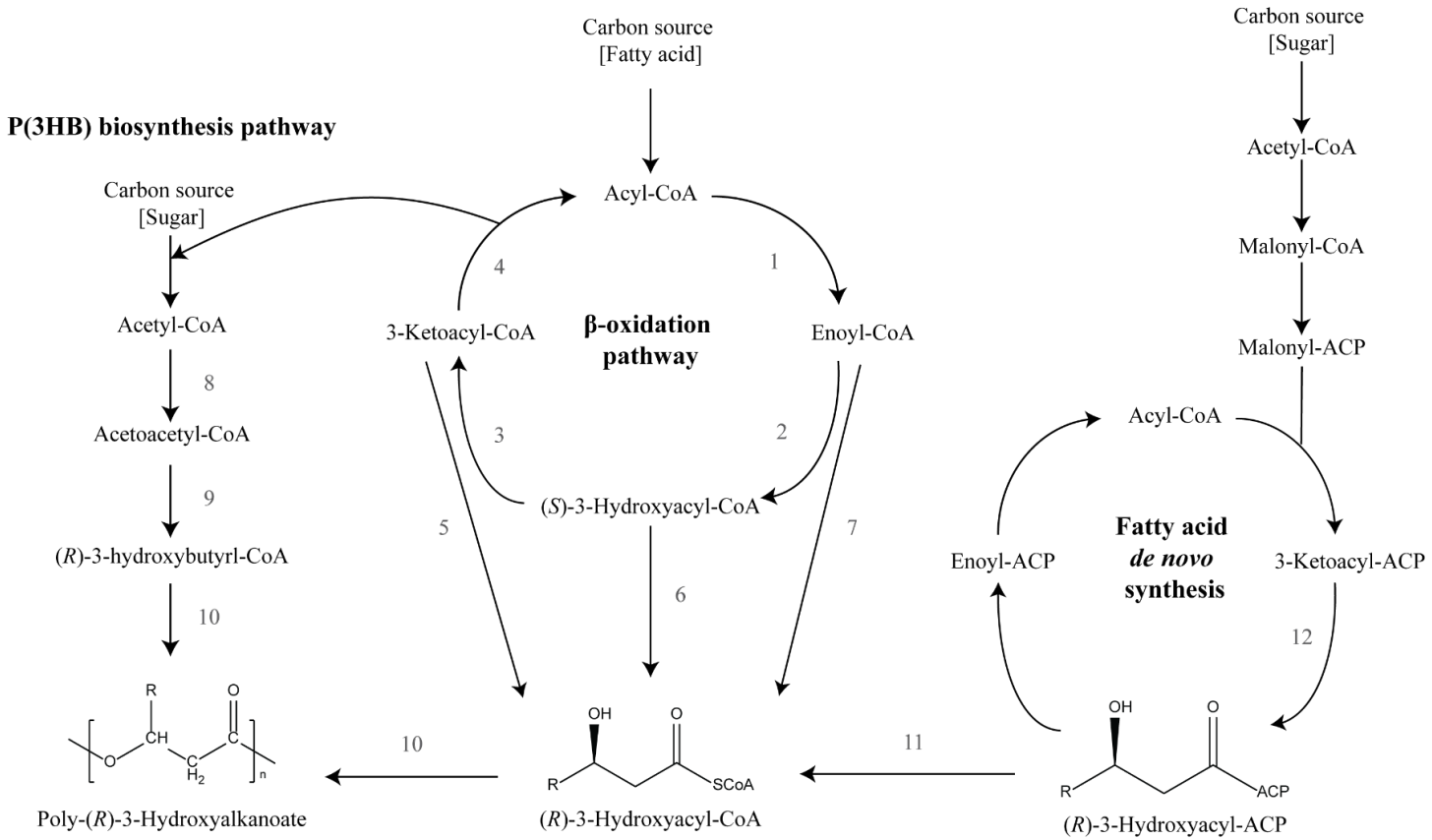


Figure 2.3: PHA biosynthesis pathways (Sudesh *et al.*, 2000a). The numbers indicate the enzyme involved in the PHA metabolic pathways. Enzymes: 1. acyl-CoA dehydrogenase; 2. enoyl-CoA hydratase; 3. 3-hydroxyacl-CoA dehydrogenase; 4. 3-ketoacyl-CoA thiolase; 5. 3-ketoacyl-CoA reductase; 6. epimerase; 7. (*R*)-specific enoyl-CoA-hydratase; 8. β-ketothiolase; 9. acetoacetyl-CoA reductase; 10. PhaC; 11. (*R*)-3-hydroxyacyl-ACP-CoA transferase; 12. ketoacyl-ACP-reductase.

## 2.5 PHA synthase (PhaC)

PhaC is often known as PHA polymerase, which catalyses the polymerisation of hydroxyacyl-coenzyme A (HA-CoA) derived from intermediates of various pathways into water-insoluble PHA polymer with the release of CoA (Rehm, 2003). Genes encoding these synthases have been identified and isolated from numerous bacteria species, for instance, *Pseudomonas* spp., *Bacillus* spp., and *Rhodococcus* spp. (Shamala *et al.*, 2003; Tajima *et al.*, 2003; Song *et al.*, 2008; Trakunjae *et al.*, 2021). Generally, PhaCs can be categorised into four distinct classes based on their structure, substrate specificity, and subunits.

Class I and Class II PhaCs are similar in that they both form homodimers but differ in their substrate specificities. Class I PhaCs only polymerise SCL-PHA substrates, while Class II PhaCs prefer MCL-PHA substrates. The molecular mass of Class I PhaCs is about 63 to 73 kDa, whereas the molecular mass of Class II synthases is about 60 to 65 kDa (Rehm *et al.*, 1999).

Class III and Class IV PhaCs are both classes of synthases that prefer polymerising SCL-PHA substrates and consist of two different types of subunits forming heterodimers (Tsuge *et al.*, 2015). The two subunits of Class III PhaCs are composed of PhaC and PhaE, which form the PhaEC complex. The molecular mass of the PhaC subunit is about 39 to 53 kDa, while that of the PhaE subunit is about 20 to 43 kDa. As for Class IV PhaCs, they are composed of two different subunits, PhaC and PhaR. The molecular mass of the PhaC subunit is approximately 40 kDa and is larger than the PhaR subunit, which is about 20 kDa.

However, in Class I PhaCs, there are some PhaCs that are exceptions as they can incorporate both SCL-PHA and MCL-PHA, such as PhaC from *Aeromonas*

*caviae* (PhaC$_{Ac}$), *Chromobacterium* sp. strain USM2 (PhaC$_{Cs}$) and *R. aethirivorans* I24 (PhaC$_{Ra}$) (Doi *et al.*, 1995; Fukui *et al.*, 1997; Bhubalan *et al.*, 2011; Budde *et al.*, 2011). For instance, PhaC$_{Ac}$ was able to produce P(3HB-*co*-3HHx) copolymer with up to 22 mol% of 3HHx when supplied with the suitable substrate (Fukui *et al.*, 1997; Chee *et al.*, 2012). PhaC$_{Cs}$ has a broad range of substrate specificity where the PhaC$_{Cs}$ can produce not only P(3HB-*co*-3HHx) copolymer but also terpolymer P(3HB-*co*-3HV-*co*-3HHx) (Bhubalan *et al.*, 2010; Bhubalan *et al.*, 2011).

### 2.5.1 PHA synthase from *Rhodococcus aetherivorans* I24

*R. aetherivorans* I24 was reported to have two *phaC* genes, *phaC1*$_{Ra,}$ and *phaC2*$_{Ra}$ (Budde *et al.*, 2011). Budde *et al.* (2011) reported that both the PhaCs can incorporate a wide range of 3HHx monomer fraction. For instance, *phaC1*$_{Ra}$ can incorporate from 1.5 to 11.5 mol% 3HHx monomer fraction, whereas *phaC2*$_{Ra}$ can incorporate monomer fraction of 3HHx from 1.6 to 18.9 mol% in the presence of different carbon sources such as palm oil, hexanoate, and octanoate. These two PhaCs were also grouped with Class I PhaCs, which are exceptions where they can also incorporate both SCL- and MCL-PHA monomers. Their work also showed that the *phaC2*$_{Ra}$ had a higher substrate specificity towards 3HHx compared to *phaC1*$_{Ra}$. The recombinant *C. necator* strain that expresses *phaC2*$_{Ra}$ incorporated up to 18.9 and 10.4 mol% of 3HHx monomer fractions when supplemented with hexanoate and octanoate, respectively (Budde *et al.*, 2011).

Other studies have also shown that engineered *C. necator* strains that harbour PhaC2$_{Ra}$ were capable of producing a wide range of 3HHx monomer compositions ranging from 14 to 70 mol% of 3HHx using plant oils like crude palm kernel oil, and soybean oils as the sole carbon source (Wong *et al.*, 2012; Murugan *et al.*, 2016). The

$M_w$ of the copolymer with 4 to 19 mol% of 3HHx monomer content ranged from 2.6 to 6.85 × $10^5$ Da in a decreasing trend (Murugan *et al.*, 2017). Nevertheless, the copolymer with a high amount of 3HHx monomer fraction of about 32 to 70 mol% exhibits very low $M_w$ that ranges between 1 to 3.5 × $10^5$ Da due to the increase in the fraction of 3HHx bulky monomers (Wong *et al.*, 2012).

## 2.6 Enoyl-CoA hydratase

Enoyl-CoA hydratase (PhaJ) is an enzyme that plays a crucial role in the β-oxidation pathway of fatty acid metabolism. This enzyme is responsible for catalysing the addition of a water molecule to the double bond in enoyl-CoA with *R* stereospecificity, leading to the formation of (*R*)-3-hydroxyacyl (3HA)-CoA (Waterson *et al.*, 1972; Agnihotri *et al.*, 2003).

The first monofunctional PhaJ enzyme was identified in a Gram-negative polyhydroxyalkanoate PHA-producing bacterium *A. caviae* FA440 ($PhaJ_{Ac}$), which was reported to play an essential role in providing (*R*)-3HA-CoA monomers of C4 – C6 chain length from enoyl-CoA β-oxidation (Fukui *et al.*, 1998a). This study illustrated that PhaJ is involved in the biosynthesis of an energy-storage material of PHA with MCL-PHA monomer by functioning as a monomer-supplying enzyme.

Qin *et al.* (2000) reported the conserved region among the eukaryotic hydratases. The researchers identified the motif, the hydratase 2 motif, which is characterised by the sequence [YF]-$X_{1,2}$-[LVIG][STGC]-GD$X$NP-[LIV]-H$X_5$-[AS]. Within this motif, the $Glu^{366}$ and $Asp^{510}$ are the catalytic residues essential for the enzymatic activity of the hydratases. The C-terminal sequence of $PhaJ_{Ac}$ showed a homology sequence towards the C-terminal of the yeast hydratase (Hisano *et al.*, 2003). The multiple sequence alignment of the hydratases amino acid showed that the

catalytic residues are in a conserved hydratase 2 motif (Tsuge *et al.*, 2003b; Wang *et al.*, 2013). In the study conducted by Hisano *et al.* (2003), the crystal structure analysis of the catalytic domain reveals the presence of a conserved structural fold that carries the catalytic dyad composed of an aspartic acid (D) and a histidine (H). The aspartate may function to activate a water molecule, which then attacks the substrate. At the same time, histidine may act as a proton donor to the substrate, facilitating the catalytic reaction (Wang *et al.*, 2013).

Several studies further identified and characterised PhaJ from various bacteria, including PHA-producing and non-PHA-producing bacteria. Several PhaJ were isolated from PHA-producing bacteria, including *Aeromonas hydrophila*, *P. aeruginosa*, *P. oleovorans*, *P. putida*, *C. necator*, and *Rhodospirillum rubrum* (Reiser *et al.*, 2000; Tsuge *et al.*, 2000; Tsuge *et al.*, 2003b; Lu *et al.*, 2004; Sato *et al.*, 2011; Kawashima *et al.*, 2012). Interestingly, functional PhaJs had also been successfully isolated from non-PHA-producing bacteria such as *Escherichia coli* (Park *et al.*, 2003) and recently identified PhaJ from a rubber-degrading bacteria, *Streptomyces* sp. CFMR7 (Tan *et al.*, 2022).

MaoC-like hydratase (MaoC) was found that exhibited the same activity as enoyl-CoA hydratase (PhaJ) (Park *et al.*, 2003; Wang *et al.*, 2013). Both MaoC and PhaJ enzymes catalyse the (*R*)-specific hydration of the β-oxidation intermediate, *trans*-2-enoyl-CoA to (*R*)-3-hydroxyacyl-CoA. The study done by Park *et al.* (2003) was the first study to prove that MaoC identified from *E. coli* ($MaoC_{Ec}$) is enoyl-CoA hydratase that plays a crucial role in supplying (*R*)-3-hydroxyacyl-CoA from the β-oxidation pathway to PHA biosynthesis pathway.

The $MaoC_{Ec}$ was deposited as putative aldehyde dehydrogenase in the protein database, whereby it was found that the MaoC is homologous to the $PhaJ1_{Pa}$ (Park *et*

*al.*, 2003). In the *E. coli* genome, the *maoC* gene is in an operon arrangement with the *maoA* gene. It has previously been reported that the monoamine oxidase (*mao*) operon encodes enzymes involved in the degradation of aromatic amine compounds (Steinebach *et al.*, 1996). Evidence suggests that the MaoC is involved in the biosynthesis of PHA, as it was supported by the elucidated crystal structure (Wang *et al.*, 2013). The protein forms a homodimer where each consists of a five-stranded antiparallel β-sheet and a central α-helix, folded into a Hotdog fold which is similar to the reported crystal structure of PhaJ (Hisano *et al.*, 2003; Wang *et al.*, 2013). The structural fold, catalytically important residues, and substrate binding sites are highly conserved, suggesting a common evolutionary origin for MaoC and other hydratases. The Table 2.3 summarized the PhaJ and MaoC isolated from different bacterial strains over the years.

Table 2.3: The PhaJ and MaoC isolated from different bacterial strains.

| Enoyl-CoA hydratases (PhaJ/MaoC) | Origin | Citation |
|---|---|---|
| Crotonyl-CoA hydratase | *Methylobacterium rhodesianum* | (Mothes *et al.*, 1995) |
| $PhaJ_{Ac}$ | *Aeromonas caviae* FA440 | (Fukui *et al.*, 1998b) |
| $PhaJ1_{Pa}$ | *Pseudomonas aeruginosa* DSM1707 | (Tsuge *et al.*, 2000; Tsuge *et al.*, 2003b) |
| $PhaJ2_{Pa}$ | | |
| $PhaJ3_{Pa}$ | | |
| $PhaJ4_{Pa}$ | | |
| $PhaJ_{Rr}$ | *Rhodospirillum rubrum* | (Reiser *et al.*, 2000) |
| $MaoC_{Ec}$ | *Escherichia coli* | (Park *et al.*, 2003) |
| $PhaJ1_{Pp}$ | *Pseudomonas putida* KT-2440 | (Wang *et al.*, 2010; Flores-Sánchez *et al.*, 2020) |
| $PhaJ3_{Pp}$ | | |
| $PhaJ4_{Pp}$ | | |
| $PhaJ4a_{Cn}$ | *Cupriavidus necator* H16 | (Kawashima *et al.*, 2012) |
| $PhaJ4b_{Cn}$ | | |
| $PhaJ4c_{Cn}$ | | |
| $MaoC_{Pc}$ | *Phytophthora capsica* | (Wang *et al.*, 2013) |
| $PhaJ1_{Hm}$ – $PhaJ5_{Hm}$ | *Haloferax mediterranei* | (Liu *et al.*, 2016) |
| $PhaJ_{YB4}$ | *Bacillus cereus* YB-4 | (Kihara *et al.*, 2017) |
| $PhaJ_{Ss}$ | *Streptomyces* sp. CFMR 7 | (Tan *et al.*, 2022) |

### 2.6.1 Substrate specificities of enoyl-CoA hydratase

The substrate specificities of the PhaJs are essential for determining monomer supply preference towards SCL-PHA monomer or MCL-PHA monomer to be incorporated for PHA polymerisation. The substrate specificity of PhaJs can be dependent on the source of PhaJs (Tsuge *et al.*, 2003b). For instance, catalytic efficiency toward the C6 substrate than the C4 substrate would be favourable for the supply of more (*R*)-3HHx-CoA monomer for PHA polymerisation into P(3HB-*co*-3HHx) through hydration of *trans*-2-enoyl-CoA from β-oxidation cycles.

In *P. aeruginosa* PAO1, four distinct PhaJs have been identified, and each of these enzymes showed different substrate preferences (Tsuge *et al.*, 2000; Tsuge *et al.*, 2003b). For instance, PhaJ1$_{Pa}$ showed high hydratase activities towards crotonyl-CoA (C4) and 2-hexenoyl-CoA (C6), leading to a high fraction of 3HHx with 78 mol% in the polymer. PhaJ2$_{Pa}$ and PhaJ4$_{Pa}$ showed predominant hydratase activity towards octenoyl-CoA (C8), which accumulated relatively high fractions of 3-hydroxyoctanoate (3HO) (30 to 34 mol%). PhaJ3$_{Pa}$ exhibit high hydratase activity towards C8, decanoyl-CoA (C10), and dodecenoyl-CoA (C12) substrates, which lead to the incorporation of more 3HO (35 to 43 mol%), 3HD (26 mol%) and 3-hydroxydodecanoate (3HDD) (8 to 17 mol%) in the polymer (Tsuge *et al.*, 2000; Tsuge *et al.*, 2003b). In *C. necator*, a total of 16 *phaJ* genes have been identified, but only *phaJ4a*$_{Cn}$, and *phaJ4b*$_{Cn}$ genes have demonstrated substrate affinity for MCL *trans*-2-enoyl-CoA, specifically towards C6 and C8 fatty acids that enhanced the 3HHx composition of the copolymer (Kawashima *et al.*, 2012).

A recent work done by Tan *et al.* (2022) further proved that higher specificity activity of PhaJ$_{Ss}$ towards the C6 compared to C4 substrate can significantly improve the 3HHx monomer composition up to 18 mol% of 3HHx. In a previous research

using PhaJ$_{Ac}$, the enzyme's specificity activity towards the C4 substrate was higher than the C6 substrate, and hence the 3HHx monomer composition accumulated only up to 5 mol% (Fukui *et al.*, 1998b).

## 2.7 Genetic engineering of strains for PHA production

The *C. necator* wild-type can only produce P(3HB) however, by genetic engineering techniques, the strain can be further modified and improved to produce P(3HB-*co*-3HHx). Studies about the overexpression of enoyl-CoA hydratases showed promising results for producing P(3HB-*co*-3HHx). Alternative approaches have similar purposes, as several studies were done to control the 3HHx molar compositions. The study by Mifune *et al.* (2010) showed that introducing *phaC*$_{Ac}$ derived from *Aeromonas caviae* that have substrate specificity towards 3HHx monomer into *C. necator* made the strain capable of producing P(3HB-*co*-3HHx). Another study conducted by Tan *et al.* (2020) too reported that the *phaC* gene played an important role in controlling the 3HHx molar composition in P(3HB-*co*-3HHx) by using different PhaCs including PhaC$_{BP\text{-}M\text{-}CPF4}$ and PhaC$_{Cs}$ from *Chromobacterium* sp. USM2.

Another approach done by Budde *et al.* (2011) uses the deletion of the *phaB* gene to control the molar composition of PHA. The deletion of *phaB* gene affects the supply of 3HB monomer and hence increases the 3HHx monomer composition. Other studies about the deletion of *fadB* or overexpression of *fabG* can also increase the 3HHx molar composition in P(3HB-*co*-3HHx) (Nomura *et al.*, 2008; Insomphun *et al.*, 2015). The recent study by Oh *et al.* (2024) maximized 3HHx monomer composition in the production P(3HB-*co*-3HHx) by controlling the concentration of fatty acid, which is the lauric acid without affect the cell growth.