# CHARACTERISATION OF POLYHYDROXYALKANOATE SYNTHESIS BY *Azotobacter vinelandii* Δ*Avin_16040*

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# CHARACTERISATION OF POLYHYDROXYALKANOATE SYNTHESIS BY *Azotobacter vinelandii* Δ*Avin_16040*

**by**

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| | |
|---|---|
| 1,4-BD | 1,4-butanediol |
| $^{1}$H NMR | Proton nuclear magnetic resonance |
| 3D | Three-dimensional |
| 3HB | 3-hydroxybutyrate |
| 3HB-CoA | 3-hydroxybutyryl-CoA |
| 3HB-CoA | *R*-3-hydroxybutyrl coenzyme A |
| 3HHx | 3-hydroxyhexanoate |
| 3HV | 3-hydroxyvalerate |
| 4HB | 4-hydroxybutyrate |
| 4HB-CoA | 4-hydroxybutyryl-coenzyme A |
| AM | Ashby's medium |
| ANOVA | Analysis of Variance |
| APS | Ammonium persulfate |
| BLAST | Basic local alignment search tool |
| BM | Burk's medium |
| BSA | Bovine serum albumin |

| | |
|---|---|
| C/N ratio | Carbon to nitrogen ratio |
| CCD | Central composite design |
| CME | Caprylic methyl ester |
| CPKO | Crude palm kernel oil |
| DA | DSMZ-*Azotobacter* |
| DSC | Differential scanning calorimetry |
| DTNB | 5,5'-dithio-bis-(2-nitrobenzoic acid) |
| EDP | Entner-Doudoroff pathway |
| FabG | 3-ketoacyl-ACP reductase |
| FID | Flame ionization detector |
| GC | Gas chromatography |
| GPC | Gel permeation chromatography |
| HAME | Hydroxyalkanoate methyl ester |
| HMDS | Hexamethyldisilazane |
| LB | Luria Bertani |
| MAM | Modified AM |
| mcl | Medium-chain-length |

| | |
|---|---|
| MDA | Modified DA |
| MIT | Massachusetts Institute of Technology |
| MMPHA | Minimal medium PHA |
| Na-4HB | Sodium 4-hydroxybutyrate |
| Na-5HV | Sodium 5-hydroxyvalerate |
| NCBI | National Center for Biotechnology Information |
| ND | Not determined |
| NMG | 1-nitroso-3-nitro-1-methylguanidine |
| NMR | Nuclear magnetic resonance |
| NPCM | Non-PHA cellular mass |
| NR | Nutrient rich |
| OD | Optical density |
| OFAT | One factor at a time |
| P(3HB) | Poly-3-hydroxybutyrate |
| P(3HB-*co*-3HV) | Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) |
| P(3HB-*co*-4HB) | Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| PHA | Polyhydroxyalkanoate |
| PhaA | 3-ketoacyl-CoA thiolase |
| PhaB | NADH/NADPH-dependent acetoacetyl-CoA reductase |
| PhaC | PHA synthase |
| PhaG | (*R*)-3HA-ACP-CoA transferase |
| PhaJ | Enonyl-CoA hydratase |
| PhaZ | PHA depolymerase |
| PP | Polypropylene |
| *proC* | Pyrroline-5-carboxylate reductase gene |
| PTFE | Polytetrafluoroethylene |
| RID | Refractive index detector |
| RSM | Response surface methodology |
| SCF | Supercritical fluid |
| scl | Short-chain-length |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Scanning electron microscopy |

| | |
|---|---|
| s-layer | Surface layer |
| TCA | Tricarboxylic acid |
| TE | Trace elements |
| TEM | Transmission electron microscopy |
| TEMED | Tetramethylethylenediamine |
| TNB | 5-thio-2-nitrobenzoic acid |
| UHMW | Ultra-high-molecular-weight |
| W | Weight of sample |
| γ-BL | Gamma-butyrolactone |

## LIST OF UNITS AND SYMBOLS

| | |
|---|---|
| % | Percent |
| % v/v | Percent volume per volume |
| % w/v | Percent weight per volume |
| Δ | Deletion |
| °C | Degree Celcius |
| °C/min | Degree Celcius per minute |
| µg/L | Microgram per liter |
| µL | Microliter |
| µm | Micrometer |
| µmol | Micromole |
| $a_0$ | Constant coeffiecient (general average) |
| $a_1$, $a_2$, $a_3$ | Constant for linear coeffiecient |
| $a_{11}$, $a_{22}$, $a_{33}$ | Constant for quadratic coeffiecient |
| $a_{12}$, $a_{13}$, $a_{23}$ | Constant for interaction coeffiecient |
| $A_{CME}$ | Area of GC peak for CME |
| $A_{HA}$ | Area of GC peak for monomer |

| | |
|---|---|
| $A_{HA1}$ | Area of GC peak for first monomer in copolymer |
| $A_{HA2}$ | Area of GC peak for second monomer in copolymer |
| bp | Base pairs |
| cm | Centimeter |
| Đ | Polydispersity index |
| Da | Dalton |
| g | Gram |
| *g* | Gravity |
| ɣ | Gamma |
| g/g | Gram/gram |
| g/L | Gram per liter |
| GPa | Gigapascal |
| k | Constant for monomer |
| K | GC constant |
| kbp | Kilo base pairs |
| kDa | Kilo Dalton |
| kGy | Kilogray |

| | |
|---|---|
| kPa | Kilopascal |
| L | Liter |
| M | Molar |
| mg | Milligram |
| mg/mL | Milligram per milliliter |
| MHz | Mega Hertz |
| mL | Milliliter |
| mL/min | Milliliter per minute |
| mm | Millimeter |
| mM | Millimolar |
| $M_n$ | Number average molecular weight |
| mol % | Mole percent |
| MPa | Megapascal |
| $M_W$ | Weight-average molecular weight |
| N | Normality |
| nm | Nanometer |
| psi | Pound-force per square inch |

| | |
|---|---|
| $R^2$ | Coefficient of regression |
| rpm | Revolutions per minute |
| *Tc* | Crystallisation temperature |
| *Tg* | Glass transition temperature |
| $T_m$ | Melting temperature |
| U | Unit of enzyme |
| U/mg | Unit per milligram |
| V | Volt |
| $X_1$, $X_2$, $X_3$ | Three variables (C: N ratio, cultivation duration, agitation speed) |
| Y | Predicted response (PHA concentration) |
| α | Alpha |
| β | Beta |

**PENCIRIAN SINTESIS POLIHIDROKSIALKANOAT OLEH**

***Azotobacter vinelandii* ΔA*vin_16040***

## ABSTRAK

Polihidroksialkanoat (PHA) dikenali sebagai termoplastik biodegradasi yang boleh diperbaharui dan mempunyai potensi untuk menggantikan plastik sintetik berasaskan petroleum kerana mempunyai sifat yang serupa. *Azotobacter vinelandii*, sejenis bakteria Gram-negatif daripada tanah, adalah salah satu mikrob yang boleh menghasilkan PHA. Banyak penyelidikan telah dijalankan untuk meningkatkan penghasilan atau nilai PHA. Kajian ini melibatkan satu strain jenis liar, *A. vinelandii* ATCC 12837, dan satu mutan delesi daripada *A. vinelandii* yang tiada atau kekurangan lapisan-S, dinamakan sebagai Δ*Avin_16040*, telah digunakan untuk menghasilkan PHA. Pengumpulan PHA ini telah dinilai di bawah parameter yang berbeza dan dibandingkan dengan strain jenis liar. Mutan menghasilkan jumlah PHA 3 kali ganda lebih tinggi berbanding dengan strain jenis liar. Hal ini mungkin disebabkan oleh ketiadaan lapisan-S di sekeliling sel. Penghasilan PHA daripada strain mutan secara optimum telah menunjukkan penambahan masing-masing sebanyak 6 % dan 39.5 % bagi berat kering sel dan jumlah PHA. Sifat poli(3-hidroksibutirat) [P(3HB)] yang dihasilkan oleh dua strain *A. vinelandii* adalah serupa. Di samping itu, bakteria ini diketahui dalam penghasilan PHA yang mengandungi 3-hidroksibutirat (3HB) atau campuran 3HB dan 3-hidroksivalerat (3HV). Menariknya, kajian ini telah mengesankan penghasilan monomer 4-hidroksibutirat (4HB) bagi pembentukan poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] daripada kedua-duanya strain jenis liar dan mutan apabila sumber karbon yang berkaitan dengan struktur

digunakan. Penemuan ini telah meningkatkan nilai polimer yang dihasilkan kerana sifat PHA yang mengandungi 4HB berguna dalam bidang perubatan. Gen sintase PHA kemudian diklon ke dalam *Cupriavidus necator* PHB$^{-}$4 dan *Cupriavidus necator* Re2058 yang biasanya digunakan untuk penghasilan PHA. Langkah ini dilakukan untuk menentukan spesifisiti substrat gen sintase PHA. Kedua-dua jenis *C. necator* PHB$^{-}$4 dan *Cupriavidus necator* Re2058 boleh menghasilkan PHA yang mengandungi 10 mol% 4HB yang serupa dengan strain *A. vinelandii*. Kajian ini adalah yang pertama melaporkan penghasilan 4HB daripada strain *A. vinelandii*, strain transformasi *C. necator* PHB$^{-}$4 dan *Cupriavidus necator* Re2058 mengandungi gen sintase PHA daripada strain *A. vinelandii*. Ketulenan PHA yang lebih tinggi telah dihasilkan daripada strain mutan berbanding strain jenis liar melalui pemulihan dengan penggunaan bahan kimia. Hal ini menjelaskan bahawa kekurangan lapisan-S telah memudahkan pencernaan sel dan pemulihan PHA.

**CHARACTERISATION OF POLYHYDROXYALKANOATE SYNTHESIS BY *Azotobacter vinelandii* Δ*Avin_16040***

## ABSTRACT

Polyhydroxyalkanoate (PHA) is a biodegradable and renewable thermoplastic that has potential to replace synthetic petroleum-based plastic due to their similar properties. *Azotobacter vinelandii*, a Gram-negative soil bacterium, is one of the microbes that can produce PHA. Many studies have been carried out to increase the production or value of PHA. In this study, a wild type strain, *A. vinelandii* ATCC 12837, and a deletion mutant of *A. vinelandii* that without or lacks the S-layer, designated as Δ*Avin_16040,* were used to produce PHA. The PHA accumulation was evaluated under different parameters and compared with wild-type strain. Around 3-fold higher amount of PHA was obtainable by the mutant strain compared to the wild type strain and the absence of the S-layer surrounding the cell was believed to played important role on higher PHA production. Optimisation on PHA production by the mutant strain had shown increment of 6 % and 39.5 % for cell dry weight and PHA concentration, respectively. The properties of poly(3-hydroxybutyrate), [P(3HB)] between two *A. vinelandii* strains were similar in terms of molecular weight, thermal and tensile properties. Besides, this bacterium is known to produce PHA containing 3-hydroxybutyrate (3HB) or mixture of 3HB and 3-hydroxyvalerate (3HV). Interestingly, in this study, 4-hydroxybutyrate (4HB) monomer was discovered to be incorporated for formation of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] by both the wild type and mutant strains when structurally-related carbon source was provided. This finding had increased the value of the polymer produced because PHA

with 4HB is useful in medical field due to its properties. The PHA synthase gene was then cloned into *Cupriavidus necator* PHB$^{-}$4 and Re2058 which are commonly used for PHA accumulation. This step was performed to determine the substrate specificity of the PHA synthase gene. Both *C. necator* PHB$^{-}$4 and Re2058 could produce PHA containing 4HB with similar composition to *A. vinelandii* strains which was around 10 mol %. This is the first study to report 4HB production using *A. vinelandii* strains as well as *C. necator* PHB$^{-}$4 and Re2058 containing PHA synthase gene of *A. vinelandii*. Higher PHA purity was obtained by the *A. vinelandii* mutant strain compared to that of the wild type strain through chemical digestion treatment suggesting that the cell of mutant strain could be easily digested to release PHA due to lack of S-layer.

# CHAPTER 1: INTRODUCTION

*Azotobacter vinelandii* is a Gram-negative and nitrogen-fixing soil bacterium. It is an interesting bacterium that can generate multi-products such as polyhydroxyalkanoate (PHA), extracellular alginate, siderophore, plant growth hormones and antibiotics (Gonzalez-Lopez et al., 1986; Page & Knosp, 1989). Its cell wall structure is complex and consists of surface layer (S-layer). This layer is reported to attach the bacterial cell towards plant cell (Merrigan et al., 2013; Liew et al., 2015). According to previous study, a deletion strain of *A. vinelandii* without S-layer was constructed to evaluate the role of the layer in bacteria-plant attachment (Liew et al., 2015). Absence of this layer was found to reduce the affinity of bacterial cells towards plant. The different characteristics between the *A. vinelandii* wild type strain and deletion mutant strain is unknown and required to be known. One of the ways to characterise these two different strains is through PHA biosynthesis since both bacterial strains can accumulate PHA. PHA is known as one of the potential alternatives to replace synthetic plastic due to its similar properties to synthetic petrochemical plastic such as polypropylene and polyethylene (Doi, 1990; Dong & Sun, 2000; Flieger et al., 2003). Plastic is a petroleum-based synthetic material that is widely used in different fields such as packaging, construction and building, mobility and transport, sport and leisure. Increasing human population has increased the demand of plastic usage but the natural resources are getting limited. Besides, these plastics are non-degradable materials and they take years to be decomposed. Accumulation of plastic debris has caused severe problems on environment that in turns lead to pollution. From last decade, many alternative materials including PHA have been discovered, synthesized and developed to replace the conventional synthetic chemical plastics.

PHA is a biodegradable thermoplastic that is accumulated by various microbes in their bodies as intracellular carbon and energy storage under conditions of excess in carbon source and limitation of other nutrients (Anderson & Dawes, 1990). Common PHA-producing strains are *Cupriavidus necator*, *Alcaligenes latus*, *Bacillus megaterium* and *Pseudomonas oleovorans*. PHA shares similar properties with synthetic plastic and thus has high possibility to replace synthetic plastic in future. There are now more than 150 types of monomers that have been discovered including the most general types 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB) and 3-hydroxyhexanoate (3HHx) (Steinbüchel & Lütke-Eversloh, 2003). Combination of different monomers and compositions will change the properties of the polymer. In other words, we can adjust our desired properties of bioplastic by changing the monomers and their compositions. However, the cost of producing this bioplastic including the carbon substrate and its downstream processes is too high. Therefore, many studies have been carried out to find out low-cost carbon substrate or renewable waste as carbon source for PHA production and enhancement of PHA recovery.

Higher PHA accumulation by the *A. vinelandii* deletion mutant strain compared to that of wild type strain is hypothesised. Absence of S-layer may increase the uptake of carbon and other nutrients or decrease the utilisation of nutrients for S-layer protein formation. Besides, the PHA recovery is also hypothesised to be improved as the absence of S-layer may result in thinner cell wall and easier solubilisation of cell materials. By the way, this bacterium was reported to produce only poly-3-hydroxybutyrate [P(3HB)] homopolymer and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] copolymer (Ryu et al., 2008). However, there is lack of study on the PHA synthase (PhaC) of this bacterium as this enzyme plays a significant role for production of different PHA monomers due to its substrate

specificity. Different monomer incorporation could alter the properties of PHA that lead to more valuable PHA and could be applied in various applications. Apart from that, the PHA recovery processes from the mutant strain should be enhanced due to absence of S-layer. It is of hope that the absence of S-layer could provide an insight for higher PHA production and efficient recovery method so that the cost for PHA would be reduced.

### 1.1 Objectives

In short, the main objective of this study is to characterise *A. vinelandii* Δ*Avin_16040* mutant strain through PHA production and recovery. The specific objectives in current study are:

1) To evaluate and optimise the production of PHA from *A. vinelandii* Δ*Avin_16040* mutant strain.
2) To compare the characteristics of PHA produced from both wild type and mutant strains of *A. vinelandii*.
3) To express PHA synthase from *A. vinelandii* Δ*Avin_16040* mutant strain in *C. necator* PHB$^{-}$4 and Re2058.
4) To investigate and compare the recovery of PHA produced by *A. vinelandii* wild type and mutant strains.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 *Azotobacter vinelandii*

*A. vinelandii* is a Gram-negative soil bacterium that can fix atmospheric nitrogen. Nitrogen fixation is controlled by nitrogenase complexes that can be inhibited by oxygen (Kennedy & Toukdarian, 1987). At the same time, this bacterium is an obligate aerobe and thus requires oxygen for growth. High respiration rate of this bacterium has maintained the function of nitrogenase complexes and acquired sufficient oxygen for cell metabolism. Increase of oxygen surrounding the bacterial cells will inactivate their nitrogenase complex. To ensure the activation of nitrogen complex, oxygen level surrounding the bacterial cells should be controlled. *A. vinelandii* has the ability to produce polyhydroxyalkanoate (PHA), alginate, phenolic lipid, siderophores, plant growth antibiotics and hormones (Gonzalez-Lopez et al., 1986).

*A. vinelandii* can survive under environmental stress due to its mechanisms. The life cycle of *A. vinelandii* is mainly divided into vegetative life cycle and encystment (Sadoff, 1975). At vegetative stage, the bacterium is observed in rod shape. Encystment begins by rounding the bacterial cells and rapidly producing PHA. Nitrogen fixation cannot be performed during encystment. Outer coating of cells is built and composed of alginate. Exine is formed as dense compact coat while viscous material that is known as intine is then developed between exine and cell wall. The mature cyst is formed to resist desiccation and chemicals by increasing intine's size and decreasing size of central body (Socolofsky & Wyss, 1962). Formation of cyst is generally due to deficiency of carbon in surrounding environment.

### 2.1.1 Ability of *A. vinelandii* to produce PHA

Both PHA and alginate can be produced by *A. vinelandii* and they will compete for carbon substrate and other nutrients for their synthesis. The pathways of *A. vinelandii* for PHA and alginate production are demonstrated in Figure 2.1. The PHA metabolic pathway of *A. vinelandii* involves three main reactions for PHA generation by using 3-ketoacyl-CoA thiolase (PhaA), NADH- or NADPH- dependent acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC). The *phbBAC* operon of *A. vinelandii* is composed of genes to produce PHA (Peralta-Gil et al., 2002). Different mutants were constructed by mutating *phaA*, *phaB* and *phaC* to evaluate the importance of these individual genes on PHA accumulation (Segura et al., 2000; Segura et al., 2003a). The results showed that no PHA was produced when the *phaB* and *phaC* were mutated while mutant of *phaA* managed to accumulate low amount of PHA due to presence of other ketothiolases. This bacterium was also reported to have PHA depolymerase (PhaZ) that can degrade PHA. Utilisation of glucose as carbon substrate induces catabolic pathway that is known as Entner-Doudoroff pathway (EDP) to produce intermediates such as glyceraldehyde-3-phosphate and pyruvate. These two intermediates can either form acetyl-CoA through catabolism or fructose-6-phosphate *via* gluconeogenesis (Beale & Foster, 1996).

Different carbon substrates can be consumed by *A. vinelandii* to produce PHA such as P(3HB) and P(3HB-*co*-3HV). Around 2.5 g/L and 6.8 g/L of P(3HB) were accumulated by cultivating *A. vinelandii* UWD using 20 g/L of glucose and sugar beet molasses, respectively (Page, 1989). By conducting fed-batch cultivation, *A. vinelandii* UWD and *A. vinelandii* mutant OPNA strain could produce approximately 25 g/L of P(3HB) (Page & Cornish, 1993; García et al., 2014). P(3HB-*co*-3HV) with different 3HV molar fractions was produced from *A. vinelandii* UWD using glucose

and different concentrations of precursors such as propionate, heptanoate, nonanoate or trans-2-pentenoate (Page et al., 1992).

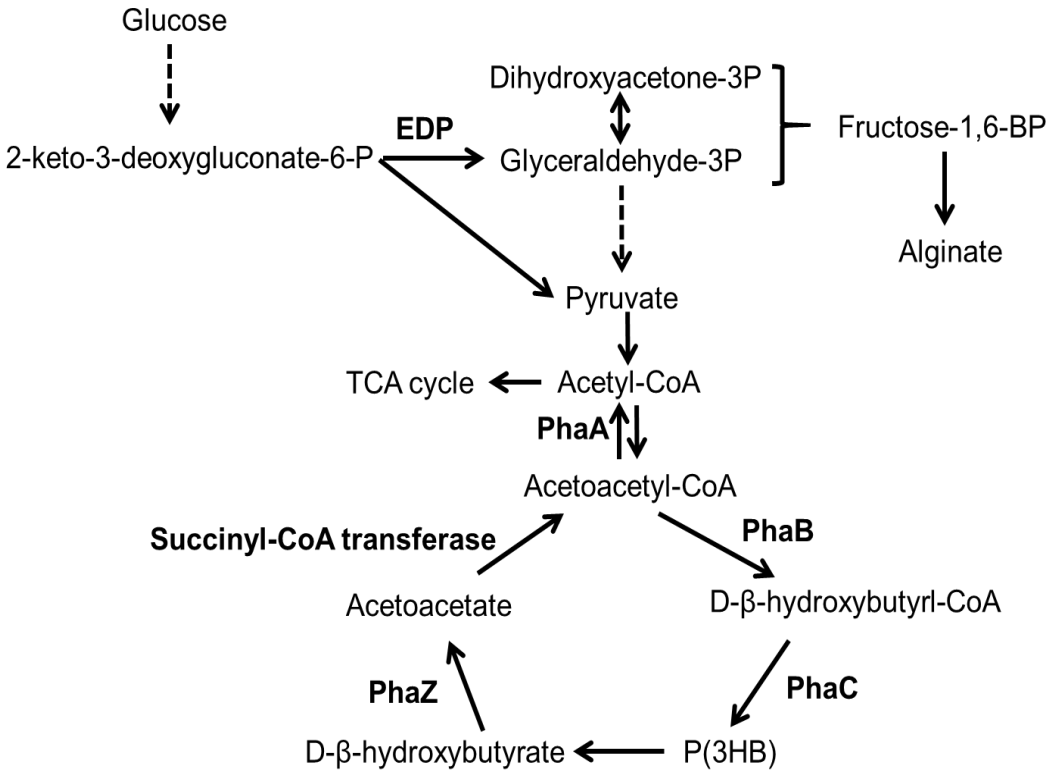



Figure 2.1: Glucose catabolism, alginate and P(3HB) biosynthesis pathways in *A. vinelandii* (Segura et al., 2003b). Abbreviated EDP is Entner-Doudoroff pathway and TCA is tricarboxylic acid.

### 2.1.2 Construction of *A. vinelandii* Δ*Avin_16040* mutant strain

A deletion mutant, *A. vinelandii* Δ*Avin_16040* mutant strain was constructed to determine the role of S-layer towards plant root adherence (Liew et al., 2015). Wild type of *A. vinelandii* was surrounded by surface layer (S-layer) and homologous replacement was conducted to delete *Avin_16040* gene that generated protein of S-layer. The upstream and downstream gene sequences of *Avin_16040* gene as well as

gene sequence of kanamycin resistance were amplified with specific primers containing known sequences of restriction enzymes. Digestion and fusion of these DNA fragments were performed. Next, ligation of the fused DNA and pDM4 was conducted to form pDM4-*16040*m. This replacement vector was transformed into *A. vinelandii* ATCC 12837 and the colonies that could grow in agar supplementing kanamycin were selected. These colonies were further examined using polymerase chain reaction (PCR) to validate absence of *Avin_16040* gene. This deletion mutant was then found to decrease cellular adhesion towards plant roots due to absence of S-layer.

**2.2 S-layer protein**

S-layer is the outermost layer of many bacteria and archaea including *A. vinelandii*, *Geobacillus stearothermophilus*, *Clostridia*, *Lactobacilli*, *Bacillus anthracis*, *Aeromonas hydrophila* and *Delftia acidovorans* (Sára & Sleytr, 2000). This layer is arranged in regular order and attached to lipopolysaccharide of bacterial outer membrane. This phenomenon usually happens on Gram-negative bacterium. S-layer is mostly composed of protein or glycoprotein subunits to form complex and porous lattice. Previous publications show that the molecular masses of this protein are in range from 25 to 200 kDa and the thickness of the S-layer is with range from 5 to 20 nm. This layer maintains the survival of bacterial cell from environmental stress and desiccation (Bingle et al., 1984; Ristl et al., 2010; Pavkov-Keller et al., 2011). It is important to enable the adhesion between microbial cells and other surfaces such as plant cells by providing surface charge on bacterial cell, production of biofilm and pathogenesis of bacteria (Sleytr et al., 1997). Since *A. vinelandii* is a soil bacterium

that can carry out nitrogen fixation, S-layer may play a vital role on bacterium-plant attachment. Construction of the deletion mutant had proven that S-layer was important for cell-adhesion towards plant (Liew et al., 2015).

## 2.3 Polyhydroxyalkanoate (PHA)

PHA is an attractive biomaterial that is produced by numerous microorganisms. The entire process of PHA polymerisation happens in microbial cytoplasm under conditions of excess carbon source and depletion of other nutrients such as nitrogen, oxygen, magnesium, sulfur and phosphorus. Hence, this polymer is known as naturally-occurring polymer or bioplastic (Sudesh & Iwata, 2008). This polymer is stored as carbon and energy reserve material in the form of intracellular granule. The granule is consumed by bacterium itself when there is lack of food source from the surrounding environment (Anderson & Dawes, 1990). It is a hydrophobic polymer that cannot be dissolved or solubilised in water. This polymer can be dissolved in chlorinated solvents such as chloroform. Around 0.2 to 0.5 μm of PHA granules that are surrounded by membrane with thickness of around 2 nm can be observed under phase contrast light microscope or electron microscope (Lundgren et al., 1964). The structure of PHA is made up of one hydroxyl group and one carboxyl group as illustrated in Figure 2.2.

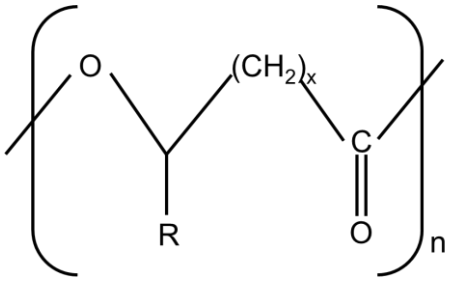



Figure 2.2: Chemical structure of PHA. Both ‘x’ and ‘n’ represent number of repeating units and ‘R’ represents side chain of the monomer (Lee, 1996)

The PHA produced has a relatively high molecular weight and shares similar properties to synthetic plastic such as polyethylene and polypropylene (Madison & Huisman, 1999). One of the advantages of the polymer is its biodegradability that this polymer can be hydrolysed enzymatically by either intracellular or extracellular depolymerase (Guérin et al., 2010). PHA is a semi-amorphous polymer that degradation of polymer is preferably taking place on amorphous part than crystalline part. Around 20 times higher of degradation rate was demonstrated on amorphous part of poly-3-hydroxybutyrate [P(3HB)] compared to its crystalline part. This study showed that the degradation rate is affected by crystallinity of polymer (Kumagai et al., 1992). Under aerobic condition, the polymer is degraded into carbon dioxide and water whereas the polymer is broken down into methane under anaerobic condition. Besides, different and wide renewable carbon resources can be utilised by microorganisms to accumulate PHA. The examples of renewable carbon sources include sugar, starch, plant oil and activated sludge. Hence, this natural bioplastic is a potential candidate to replace synthetic chemical-based plastic and ensure sustainability of environment.

P(3HB) was first discovered by a microbiologist from Paris named Maurice Lemoigne in year 1925 (Lemoigne, 1926). Subsequently, a new heteropolymer with

3HV and other medium-chain-length (mcl) monomers was found out by Wallen and Rhoweddery from activated sewage sludge (Wallen & Rohwedder, 1974). According to Findlay and White, the polymer extracted from marine sediments spotted at least 11 other types of short-chain PHA monomers besides 3HB and 3HV (Findlay & White, 1983). PHA production from *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus*) was widely studied by cultivating the bacterium in media consisting of diverse carbon sources. Incorporation of other monomers to form copolymers such as poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was successfully demonstrated (Doi et al., 1988; Kunioka et al., 1989). Poly(3-hydroxyoctanoate) was reported to be produced by growing *Pseudomonas oleovorans* ATCC 29347 (De Smet et al., 1983). Subsequent studies for mcl-PHA production were carried out by cultivating the same strain utilising *n*-alkanes, *n*-alkanoic acids and *n*-alcohols as carbon source (De Smet et al., 1983; Lageveen et al., 1988). By the way, photosynthetic bacterium, *Rhodospirillum rubrum*, was found out to successfully produce PHA with 4 to 6 carbon atoms by utilising alkanoic acid whereas *P. aeruginosa* and other *Pseudomonas* species was reported to accumulate polymer that was composed of 3-hydroxydecanoate (Brandl et al., 1989; Timm & Steinbüchel, 1990). Up till now, more than 150 types of different monomers have been discovered to be polymerised into PHA turning out to various properties.

### 2.4 Types of polyhydroxyalkanoate and its biosynthesis pathways

PHA is basically divided into two types which are short-chain-length (scl) PHA and medium-chain-length (mcl) PHA. Scl-PHA is formed by 3 to 5 carbon atoms while mcl-PHA is composed of 6 to 14 carbon atoms. Polymerisation of monomers can

produce either homopolymer or heteropolymer (copolymer) (Madison & Huisman, 1999; Nomura et al., 2004). The most common generated homopolymer by microbes is P(3HB) and incorporation of other monomers such as 4HB, 3HV and 3HHx to form copolymers by adding structurally-related carbon sources. These polymers are accumulated based on their specific metabolic pathways and enzymes.

According to Figure 2.3, three main pathways for PHA biosynthesis are established. Pathway I is the most general pathway for 3HB production while pathways II and III are known to produce mcl-PHA. For pathway I, condensation of two acetyl-CoA molecules is assessed by 3-ketoacyl-CoA thiolase (PhaA) to form acetoacetyl-CoA molecule. This acetoacetyl-CoA is then reduced to (*R*)-3HB-CoA by NADH- or NADPH- dependent acetoacetyl-CoA reductase (PhaB) and subsequent polymerisation is accomplished to form PHA by PHA synthase (PhaC). Both pathway II and III supply (*R*)-3HA-CoA monomers to polymerise into PHA by PhaC. For pathway II, the fatty acids are utilised to form enonyl-CoA followed by converting into (*R*)-3HA-CoA monomers by enonyl-CoA hydratase (PhaJ). For pathway III, sugar or other carbon sources is used for fatty acid biosynthesis and 3-ketoacyl-ACP is converted into (*R*)-3HA-ACP by 3-ketoacyl-ACP reductase (FabG). (*R*)-3HA-CoA monomers is formed from (*R*)-3HA-ACP by (*R*)-3HA-APC-CoA transferase (PhaG). Other pathways are also carried out to form 4-hydroxyacyl-CoA when structurally-related carbon sources such as sodium 4-hydroxybutyrate (Na-4HB) and 1,4-butanediol (1,4-BD) are utilised.

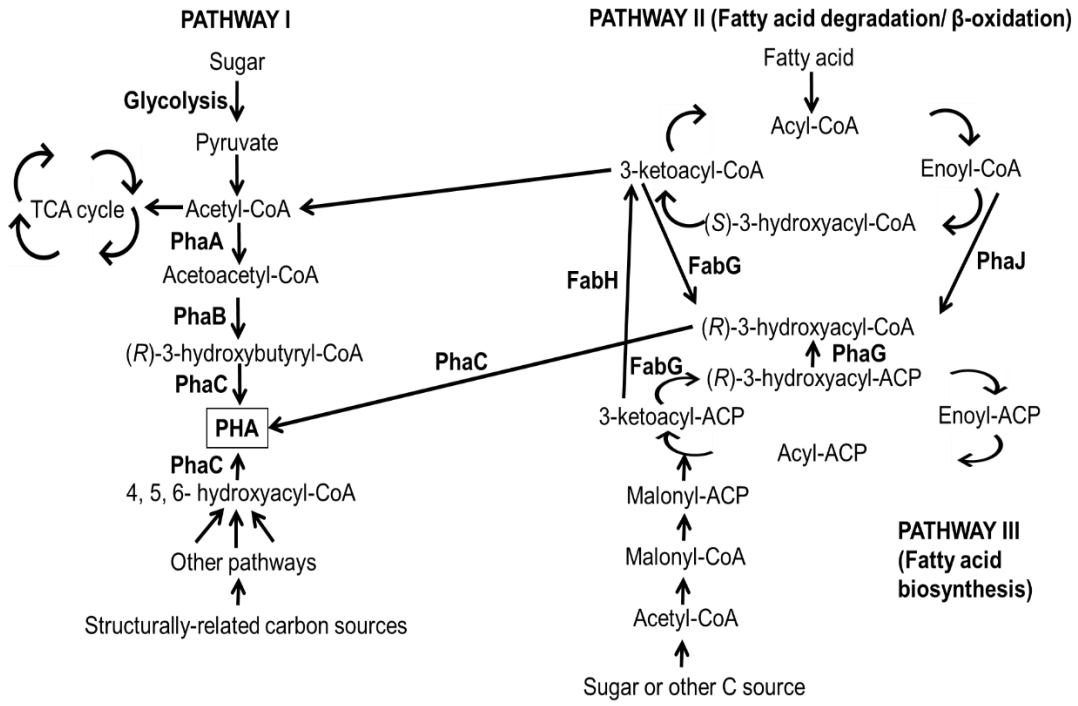



Figure 2.3: Schematic diagram of metabolic pathways for PHA biosynthesis (Sudesh et al., 2000; Lu et al., 2009).

### 2.4.1 Poly(3-hydroxybutyrate) [P(3HB)]

P(3HB) is the earliest discovered PHA from *B. megaterium*. This polymer is composed of 4 carbon atoms and a methyl group as side chain group. Chemical structure of this polymer is illustrated in Figure 2.4. This polymer was reported to have melting temperature of close to 180 °C and glass transition temperature at around 4 °C. Narrow processing window of this polymer was demonstrated as its degradation temperature is approximately at 200 °C which is nearly to melting temperature of P(3HB). This processing window has limited the applications of P(3HB). The weight-average molecular weight ($M_W$) of P(3HB) was reported in the range of $1 \times 10^4$ to $1 \times 10^7$ Da in which the latter $M_W$ belongs to ultra-high-molecular-weight (UHMW)

P(3HB). This UHMW P(3HB) was synthesised by formation and cultivation of *Escherichia coli* that contained PHA synthase from *C. necator* (Kusaka et al., 1998). The tensile strength, elongation to break and Young's Modulus of P(3HB) are 43 MPa, 5 % and 3.5 GPa whereas the same properties of UHMW P(3HB) are evaluated as 62 MPa, 58 % and 1.1 GPa (Doi, 1990; Kusaka et al., 1999). Polypropylene (PP) has comparable tensile properties as P(3HB) but much higher elongation to break (400 %). Low elongation to break of P(3HB) creates a brittle and stiff polymer. The crystallinity of P(3HB) was reported to be in range of 60 to 80 % after extraction (Holmes, 1988).

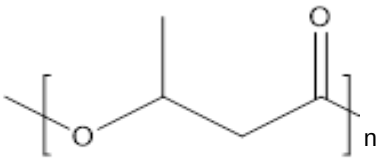



Figure 2.4: Chemical structure of P(3HB). 'n' represent number of repeating units (Doi et al., 1986).

### 2.4.2 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]

P(3HB-*co*-4HB) is a copolymer that is composed by 3HB and 4HB monomers. Unlike 3HB, 4HB is a linear structure without side chain group even though both are composed of four carbon atoms (Figure 2.5). Doi and his colleagues had discovered incorporation of 4HB monomer to form P(3HB-*co*-4HB) copolymer from *C. necator* by supplementing structurally-related carbon source which were 4-hydroxybutyric acid and gamma-butyrolactone (γ-BL). This 4HB monomer was evaluated through nuclear magnetic analysis (Doi et al., 1988). This copolymer is commonly produced

by *C. necator*, *Alcaligenes latus*, *Delftia acidovorans*, *Hydrogenophaga pseudoflava* and *Comamonas testosteronii*. Generally, 4HB monomer can only be produced under certain specific conditions. First, two different carbon sources are used in bacterial cultivation and PHA production. One of the carbon sources is mainly used to produce 3HB while another carbon source is structurally-related carbon source for 4HB production. Examples of structurally-related carbon sources are 4-hydroxybutyric acid, 1,4-BD and γ-BL (Kunioka et al., 1989; Saito & Doi, 1994). Apart from that, single structurally-related carbon source is used solely for cell growth and 4HB generation (Doi, 1990). Another alternative is by molecular modification on the bacterial cell for 4HB production using non-structurally-related carbon source. In previous study, a recombinant *E. coli* DH5α harboring the biosynthesis genes of PHA from *C. necator* and succinate degradation genes from *Clostridium kluyveri* had been constructed for 4HB generation by utilising glucose as sole carbon source (Valentin & Dennis, 1997).

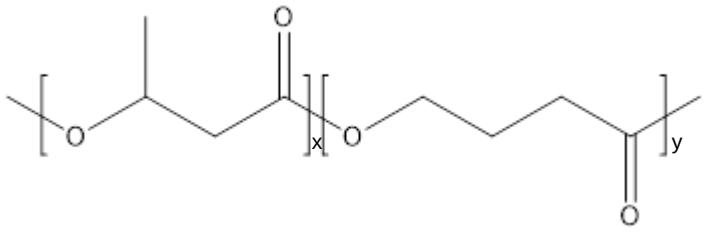



Figure 2.5: Chemical structure of P(3HB-*co*-4HB). 'x' and 'y' represent repeating unit of each monomer (Doi et al., 1988).

P(3HB-*co*-4HB) is produced when structurally-related carbon source is added. In details, 4-hydroxybutyryl-coenzyme A (4HB-CoA) is formed by conversion of 4-hydroxybutyric acid, oxidation of 1,4-BD and 1,6-hexanediol or hydrolysis of γ-BL.

This 4HB-CoA can be metabolised into either 4HB monomer or 3-hydroxybutyryl-CoA (3HB-CoA) that will further lead to 3HB monomer. Production of these two monomers has formed a random copolymer. Change of 3HB and 4HB compositions can be affected by the utilisation of carbon sources. Increment of 3HB composition but reduction of 4HB composition happens when mixture of two carbon sources is used for cell cultivation such as mixture of fructose and γ-BL. By using two structurally-related carbon sources such as combination of γ-BL and 4-hydroxybutyric acid, the intermediate produced *via* β-oxidation will hinder conversion of 4HB-CoA into 3HB-CoA that in turns raise the 4HB composition (Doi, 1990). The schematic diagram for biosynthesis pathway of P(3HB-*co*-4HB) in *C. necator* is illustrated in Figure 2.6.

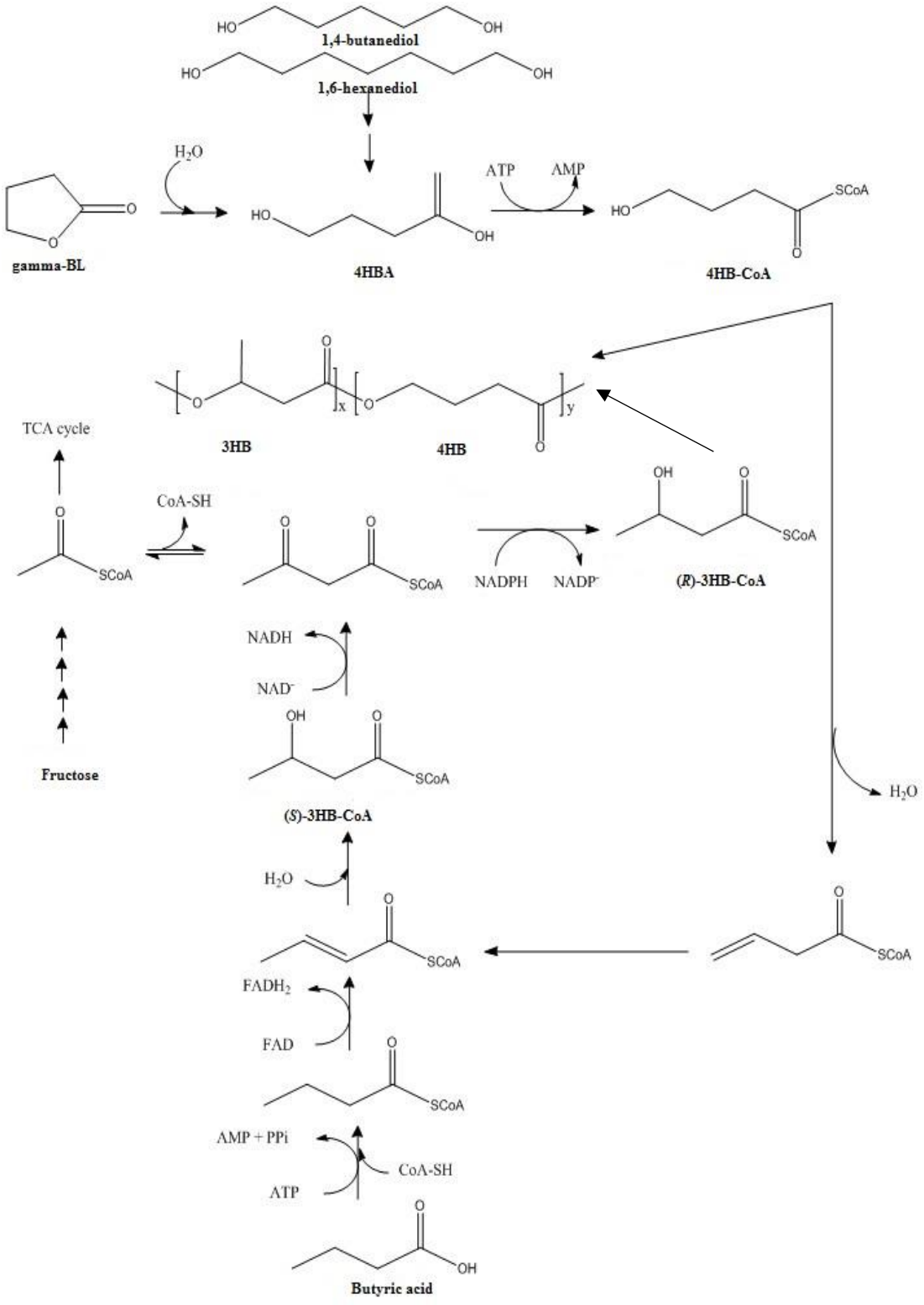



Figure 2.6: Schematic diagram for biosynthetic pathway of P(3HB-*co*-4HB) in *C. necator* (Doi, 1990).

Incorporation of this monomer has altered the polymer properties to be more suitable for applications. The crystallinities of P(3HB-*co*-4HB) with different 4HB compositions were in range of 15 to 45 %. By comparing to P(3HB) homopolymer, this copolymer was less crystalline (Saito et al., 1996). The molecular weight of P(3HB-*co*-4HB) had been analysed from certain research groups. For instance, Obata and colleagues had determined that the molecular weight of P(3HB-*co*-4HB) with 18 mol % of 4HB was around 1 million Da (Obata et al., 2013). Huong and his colleagues also had reported the molecular weight of P(3HB-*co*-4HB) containing 31 mol % of 4HB produced from *C. necator* was approximately 1 million Da (Huong et al., 2017). Besides, lower melting and glass transition temperatures are exhibited in this copolymer. For the copolymer with more than 60 mol % of 4HB, the melting temperature was around 50 °C while the glass transition temperature was within −35 to −48 °C. The number average molecular weight of P(3HB-*co*-4HB) with 17 mol % of 4HB from *C. necator* was also reported as $400 \times 10^{-3}$ Da while polymer with different 4HB molar compositions from *A. latus* was in range of 58 to $166 \times 10^{-3}$ Da (Doi, 1990; Kang et al., 1995). The tensile strength of P(3HB-*co*-4HB) with 4HB molar compositions from 0 to 64 mol % decreased to about 17 MPa but started increasing to 104 MPa by polymer with more than 64 mol % of 4HB. The elongation to break of this copolymer with high 4HB molar composition could go up to exceed 1000 % (Doi, 1990). This polymer property has increased its flexibility to form a more elastic polymer compared to P(3HB). The PHA consists of 4HB is lipase-degradable and thus, it is bioabsorbable and biocompatible. Since this copolymer is less crystalline and with linear structure, lipase could straightforwardly attach towards the copolymer for breaking down the copolymer into monomer (Nakamura et al., 1992).

### 2.4.3 Other PHAs

Besides P(3HB) and P(3HB-*co*-4HB), there are still many different types of PHA polymers. Unlike 3HB, other monomers are produced mainly due to consumption of structurally-related carbon source and the substrate specificity of PHA synthase. P(3HB-*co*-3HV) was one of the PHA copolymers and discovered in year 1983. This random copolymer composed of 3HB and 3-hydroxyvalerate (3HV) monomers. The 3HV monomer has five carbon atoms on its backbone and the ethyl group is presented as side chain group. By adding structurally-related carbon source, 3HV is produced. The structurally-related carbon source to form 3HV is carbon atoms in odd number such as propionic acid (3 carbon atoms), valeric acid (5 carbon atoms), heptanoic acid (7 carbon atoms) and nonanoic acid (9 carbon atoms) (Steinbüchel & Lütke-Eversloh, 2003). When propionic acid is utilised, propionyl-CoA is formed and further producing intermediates such as 3-ketovaleryl-CoA. Intermediates are finally converted to 3HV monomers and all these processes are catalysed by specific enzymes (Doi et al., 1987). Enhancement on the properties of this copolymer compared to P(3HB) is observed. The monomer compositions can be adjusted based on cultivation conditions and carbon sources. More than 90 mol % of 3HV can be incorporated into the polymer. This copolymer shows comparable crystallinity to P(3HB) at around 50 to 70 % (Orts et al., 1991). Co-crystallisation occurs between both P(3HB) and P(3HV) lattices. This phenomenon is known as isodimorphism. By increasing 3HV molar fraction in PHA, the melting temperature of the polymer decreases. The thermal processing window of this copolymer is enlarged at the same time because the degradation temperature of the copolymer is unchanged. Increasing 3HV content also gives rise to flexibility and toughness of the polymer by rising the elongation to break.

The tensile strength and Young's Modulus of this polymer reduce as 3HV molar fraction increases (Khanna & Srivastava, 2005).

P(3HB-*co*-3HHx) is another type of PHA. One of the monomers, 3HHx is formed by 6 carbon atoms with propyl group as side chain. This propyl group prevents co-crystallisation of P(3HB) and P(3HHx) lattices. Since this copolymer does not have isodimorphic behaviour, lower crystallinity is attained when the 3HHx content increases (Doi et al., 1995). The tensile properties of this copolymer are having similar trends as P(3HB-*co*-3HV). As 3HHx molar fraction increases, the elongation to break increases and tensile strength decreases. For instance, the tensile strength and elongation to break of P(3HB-*co*-3HHx) with 10 mol % of 3HHx are 21 MPa and 400 %, respectively. Increment of 3HHx content also reduces the melting temperature such as 5 and 10 mol % of 3HHx have melting temperatures correspondingly at around 155 °C and 127 °C (Tsuge, 2002; Loo et al., 2005).

Other than copolymer containing two different monomers, there is also presence of copolymer with more than two different monomers such as terpolymer. The examples include P(3HB-*co*-3HV-*co*-3HHx), P(3HB-*co*-3HV-*co*-4HB) and P(3HB-*co*-4HB-*co*-3HHx). The monomer compositions will alter the properties of these copolymers. PHA with 3 or more types of monomers usually results in more stable thermal properties and possesses lower crystallinity (Madden et al., 2000; Chanprateep & Kulpreecha, 2006; Xie & Chen, 2008). Different side chains are also found on PHA such as hydroxyl, carboxyl and epoxy groups. Halogenated functional group is also discovered as side chain in PHA produced by *Pseudomonas oleovorans* by using different precursors (Kim et al., 1992).

## 2.5 Enzymes for PHA biosynthesis

Several specific enzymes to catalyse PHA biosynthesis have been found along the metabolic pathways. Among all these enzymes, three of them play vital role to generate PHA which are PhaA, PhaB and PhaC.

PhaA is also known as acetyl-CoA acetyltransferase (EC 2.3.1.9). Condensation of two acetyl-CoA molecules to acetoacetyl-CoA is carried out. Generally, two types of PhaA enzymes are demonstrated based on the substrate specificity. One of the enzymes is known as biosynthetic thiolase and actively functions by substrate with 4 and 5 carbon atoms whereas another enzyme is recognized as degradative thiolase by having a wider substrate specificity that is with 4 to 16 carbon atoms (Staack et al., 1978; Haywood et al., 1988).

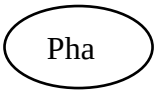
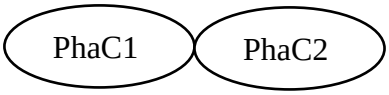
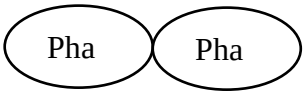
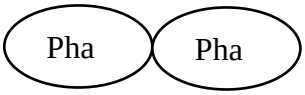
PhaB is also named as hydroxyacyl-CoA dehydrogenase (EC 1.1.1.36). Acetoacetyl-CoA is reduced to hydroxyacyl-CoA by this enzyme. Similar with PhaA, this enzyme is also divided into two kinds based on its substrate specificity. One is identified as NADH-linked acetoacetyl-CoA reductase that is active with substrate containing 4 to 10 carbon atoms while another kind is NADPH-linked acetoacetyl-CoA reductase that has substrate specificity with 4 to 6 carbon atoms.

### 2.5.1 PHA synthase (PhaC)

The key enzyme to polymerise PHA is PhaC. This synthase is under α/β hydrolase superfamily with catalytic triad containing aspartic acid, histidine and highly conserved cysteine instead of serine from lipase box (Jia et al., 2000). According to Fukui and colleagues, the synthase of *Zoogloea ramigera* was in soluble fraction when

then PHA production was low whereas the PHA production was high when the synthase was localised on PHA granule (Fukui et al., 1976). Similar results were obtained from other PHA-producing bacteria (Griebel et al., 1968; Ritchie & Dawes, 1969; Haywood et al., 1989). Four classes of PhaC can be divided based on its subunits and substrate specificity (Pötter & Steinbüchel, 2005). Class I PhaC focuses on generating scl-PHA and consists of one subunit only. Although scl-PHA is generally produced by class I PhaC, some mcl-PHAs such as 3HHx are also formed. Microbes that contain class I PhaC include *C. necator*, *Burkholderia* sp. and *Sinorhizobium melioti*. Mcl-PHA is preferably produced by class II PhaC and this PhaC also consists of two subunits (PhaC1 and PhaC2) (Matsusaki et al., 1998). *Pseudomonas aeruginosa* is the representative of this class of PhaC. However, the synthase gene of *P. aeruginosa* can be expressed to incorporate scl-monomer in PHA-negative *C. necator* mutant. Another class of PhaC is class III PhaC. This class of PhaC consists of two subunits which are PhaC and PhaE with similar sizes (Rehm & Steinbüchel, 1999). Similar as class I PhaC, class III PhaC preferably generate scl-PHA and mcl-PHA with 6 to 8 carbon atoms. The examples of class III PhaC are *Allochromatium vinosum*, *Synechocystis sp*. and *Thiocapsa pfennigii*. Class IV PhaC is composed of two subunits which are PhaC and PhaR subunits. The substrates for this class are similar with Class III PhaC. All the classes of PhaC are illustrated in Table 2.1.

Table 2.1: Classes of PhaC

| Class | Subunits | Substrate |
|---|---|---|
| I |  ~ 60 – 70 kDa | 3HA-CoA (3 to 5 carbon atoms)<br>4HA-CoA<br>5HA-CoA |
| II |  ~ 60 – 70 kDa | 3HA-CoA (≥ 5 carbon atoms) |
| III |  ~40 kDa ~40 kDa | 3HA-CoA (3 to 8 carbon atoms)<br>4HA-CoA<br>5HA-CoA |
| IV |  ~ 40 kDa ~ 22 kDa | 3HA-CoA |

## 2.6 Applications of PHA

Since petrochemical-based synthetic plastic has become limiting in source and warning due to environmental pollution, PHA is being studied to be a potential candidate to replace the conventional plastic. Similar properties of this bioplastic compared to conventional plastic have led to diverse applications. Numerous

industries have shown their attention towards bioplastic on packaging, medical and agricultural fields. Bioplastic was designed as food packaging that not only could preserve food quality under the same conditions like conventional synthetic plastic but also could be degraded naturally into carbon dioxide and water under certain conditions (Fabra et al., 2014; Scarfato et al., 2015). This degradation in turns reduces the environmental pollution since the conventional synthetic plastic is hard to be decomposed. Plastic bottles, films and bags are manufactured by processing PHA through moulding, pressing and blowing. In the 1980s, a brand name of PHA-Biopol® was commercially released. This PHA is composed of 3HB and 3HV that are further industrialised to some products such as cosmetic bottles, moisture blockage for sanitary product, disposable razors and surgical materials (Hocking & Marchessault, 1994). Strong fibres can also be produced by PHAs for textile and fisheries industries (Bugnicourt et al., 2014). Biofuel is another product that can be made by PHA *via* acid catalysed hydrolysis by converting scl- and mcl-PHAs to hydroxyalkanoate methyl ester (HAME). HAME functions as biofuel additive to increase combustion heat values (Wang et al., 2010). In addition, PHA is coated on paper to improve the water resistance and tensile strength of paper (Cyras et al., 2007). For agricultural industry, PHA is used as carrier to slowly release pesticides and herbicides to soil after microbial degradation on PHA. Conventional method to supply herbicides and pesticides requires repetitive applications of the chemicals that harm the plants while controlled release system provides sufficient herbicides and pesticides that in turns decreases impact on the plants (Voinova et al., 2009; Grillo et al., 2011).

Besides, PHA is also established as different products in medical field since the polymer is bio-absorbable. Vascular grafting is conducted to repair the damaged blood vessel while other cardiovascular products such as stents and heart valves are

fabricated (Sodian et al., 2000; Qu et al., 2005). Other products such as sutures, swabs, bone plates and spinal cages are also produced. Suture and implants that are made from P(3HB-*co*-4HB) are lipase-degradable polymer. Human can produce lipase to degrade the polymers such as sutures and implants so that they do not need to be removed (Martin & Williams, 2003). Cytotoxicity test on PHA polymer was carried out and proved that PHA did not cause harmful effect on human body after implantation (Ying et al., 2008). This PHA polymer is also used to detect lipase depolymerising activity. Ch'ng and Sudesh had newly designed a method in 2013 to observe and quantify lipase depolymerising activity by using P(3HB-*co*-4HB) thin film. Opaque spots could be seen using naked eyes on the transparent thin film with presence of lipase (Ch'ng & Sudesh, 2013). Drug delivery system is developed by PHA that has similar function as controlled released system of herbicides and pesticides. PHA is used as drug carrier by adjusting the ratio of drug and PHA polymer and size distribution of particle to control the release of drug (Pouton & Akhtar, 1996; Bansal et al., 2011). Anticancer drug is also encapsulated by PHA as carrier to treat cancers (Masood et al., 2013; Pramual et al., 2016). For tissue engineering, Lomas and co-workers have revealed that scaffold of PHA and collagen hybrid provided cell attachment after running cell viability assay (Lomas et al., 2013). Electrospun PHA fibres that contain porous structure offer attachment and regeneration of cell. Other nutrients are also blended into PHA to enhance the tissue response's effectiveness in tissue engineering aspect (Rao et al., 2010).