FABRICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE)/GELATIN BLEND SCAFFOLDS

by

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LIST OF SYMBOLS AND ABBREVIATIONS

L	Litre
G	Gram
µL	Microlitre
%	Percentage
mol%	Mole percentage
v/v	Volume per volume
w/v	Weight per volume
Mg	Microgram
°C	Degree celcius
wt%	Weight percentage
γ-BL	Gamma-butyrolactone
mL	Millilitre
Cm	Centimetre
cm^{-1}	Per centimetre
kV	Kilovolt
M_w	Molecular weight
M_n	Number average of molecular weight
M_w/M_n	Molecular weight per number average of molecular weight
PDI	Polydispersity index
FTIR	Fourier transform resonance
AFM	Atomic force microscope
SEM	Scanning electron microscope

ASTM	American Society for Testing and Materials
MEM	Minimum essential medium
FBS	Feotal bovine serum
DMSO	Dimethyl sulphoxide
CO_2	Carbon dioxide
GC	Gas chromatography
MSM	Mineral salt medium
CME	Caprylic methyl ester
Rms	Root mean square
R_q	Roughness
R_a	Mean roughness
P(4HB)	Poly(4-hydroxybutyrate)
PLA	Poly(lactic acid)
PGA	Poly(glycolic acid)
PLGA	Poly(lactic-co-glycolic acid)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB)	Poly(3-hydroxybutyrate)
PHBHV	Poly(3hydroxybutyrate-*co*-3-hydroxyvalerate)
PHBHHx	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PMS	Phenazine methosulfate
Nm	Nanometre
T25	Polystyrene flask
Da	Dalton

kDa	Kilodalton
MPa	Megapascal
HFIP	Hexafluoroisopropanol
TFE	Tetrafluoroethylene
NH_2	Amide group
OH	Hydroxy group
COOH	Carboxylic acid
Mm	Millimetre
GTA	Glutaraldehyde
cP	Centipoise
2D	Two dimensional
3D	Three dimensional
PCL	Polycaprolactone
GE	Gelatin
C=N	Imine linkage
ECM	Extracellular matrix
mL/h	Millilitre per hour
PBS	Phosphate buffer saline
cells/mL	Cells per millilitre
ANOVA	Analysis of variance
UV	Ultraviolet
PHA	Polyhydroxyalkanoate
FDA	Food and Drug Administration
Scl	Short chain length

Mcl	Medium chain length
PhaC	Pha synthase
PLLA	Poly-L-lactide
T_m	Melting temperature
T_g	Glass transition temperature
mL/min	Millilitre per minute
ω	Omega
4HB-CoA	4-hydroxybutyryl-CoA
mM	Millimolar
GPC	Gel permeation chromatography
H_2SO_4	Sulphuric acid
BSE	Bovine Spongiform Encephalopathy
DMF	Dimethylformamide
Na_2SO_4	Sodium sulphate
µm	Micrometer

FABRIKASI DAN PENCIRIAN PERANCAH CAMPURAN POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT)/GELATIN

ABSTRAK

Tujuan kajian ini dijalankan adalah untuk fabrikasi bahan baharu dengan menggabungkan bahan hidrofobik dan hidrofilik. Polimer poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) mempunyai fleksibiliti yang baik untuk diaplikasi dalam bidang perubatan, bagaimanapun, kekurangan sifat hidrofilik yang membatasi penggunaannya dalam bidang ini. Oleh sebab itu, P(3HB-*ko*-4HB) dicampurkan dengan gelatin menggunakan dua kaedah yang berbeza; pengeringan sejukbeku dan pemutaran elektro. Teknik yang pertama menghasilkan perancah berliang dengan memanipulasi pelbagai parameter termasuk komposisi monomer 4HB, kepekatan gelatin dan jumlah berat keseluruhan perancah. Morfologi permukaan perancah campuran dengan 27 mol%4HB dan 50 mol%4HB menunjukkan keliangan yang tinggi tetapi ianya menurun pada 82 mol%4HB. Kapasiti penyerapan air oleh perancah campuran didapati menurun dari 373% hingga 43% selari dengan peningkatan komposisi monomer 4HB. Kajian juga mendapati bahawa kelarutan perancah campuran dalam air telah menurun dengan peningkatan komposisi monomer 4HB, yang menunjukkan peningkatan kestabilan perancah yang lebih baik dalam larutan. Selain itu, kehadiran gelatin dalam perancah campuran telah disahkan melalui ATR-FTIR. Sementara itu, sifat hidrofilik perancah campuran menunjukkan penambahbaikan dengan kehadiran kepekatan gelatin yang berbeza. Tambahan pula, manipulasi berat keseluruhan perancah juga menyebabkan sifat perancah bertambah baik. Perancah campuran P(3HB-*ko*-50mol%4HB)/10% gelatin (50/10) dan P(3HB-*ko*-82mol%4HB)/7.5% gelatin (82/7.5) pada jumlah berat keseluruhan 0.395 g telah

menunjukkan proliferasi sel yang lebih baik berbanding dengan perancah campuran yang lain. Di samping itu, morfologi permukaan, kekasaran permukaan dan kapasiti penyerapan air oleh campuran 50/10 telah memaparkan beberapa sifat yang lebih baik berbanding dengan campuran 82/7.5. Perancah berserat lapisan demi lapisan disediakan iaitu P(3HB-*ko*-4HB)/gelatin (PG) dan P(3HB-*ko*-4HB)/gelatin/P(3HB-*ko*-4HB) (PGP), dengan menggunakan pemutaran elektro dan purata diameter serat yang diperolehi ialah 250 nm. Kedua-dua perancah lapisan berserat ini disediakan untuk mengkaji kesan lapisan yang berbeza terhadap sifat perancah berserat. Perancah lapisan berserat telah dirawat dengan wap glutaraldehid. Interaksi antara glutaraldehid dengan kumpulan amino gelatin telah dipastikan dengan menggunakan ATR-FTIR yang memaparkan puncak baharu dalam julat 1641-1631 cm^{-1}; menunjukkan tindak balas gelatin dengan glutaraldehid. Dalam pada itu, juga dapat dilihat bahawa perancah berserat PGP menunjukkan kestabilan dalam larutan sehingga 72 jam, yang mengesahkan sifat kerintangan air yang baik, manakala perancah berserat PG menunjukkan sifat hidrofilik yang lebih baik berbanding dengan P(3HB-*ko*-4HB) dan PGP. Hasil dari kajian MTS telah menunjukkan perancah berserat PG dan PGP mempunyai pertumbuhan sel yang lebih baik berbanding dengan P(3HB-*ko*-4HB). Kesimpulannya, perancah berliang dengan komposisi 50/10 dan perancah berserat PGP telah menunjukkan sifat yang sesuai untuk digunakan dalam bidang perubatan.

FABRICATION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE)/GELATIN BLEND SCAFFOLD

ABSTRACT

The aim of this study is to fabricate a new material by combining hydrophobic and hydrophilic materials. Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) has adequate flexibility for medical applications, however, its lack of hydrophilic properties limits its applications in this field. Therefore, the P(3HB-*co*-4HB) polymer was blended with gelatin using two different techniques; freeze-drying and electrospinning. The former technique can produce porous scaffolds by manipulating various parameters including 4HB monomer content, gelatin concentrations, and total weight of the scaffolds. The surface morphology results of the blend scaffolds at 27 mol%4HB and 50 mol%4HB have high porosity but the porosity of the blend scaffold decreased at 82 mol%4HB. The water absorption capacity of the blend scaffolds was found to be decreasing from 373% to 43% concurrently with the increase of the 4HB monomer. It was also discovered that the water solubility of blend scaffolds had decreased with the increase of 4HB monomer compositions, which indicates the improved stability of scaffolds in solution. Besides, the presence of gelatin in blend scaffolds was confirmed via ATR-FTIR. Meanwhile, the hydrophilicity of the blend scaffolds was improved with the insertion of different concentrations of gelatin. Furthermore, the manipulation of the total weight of the scaffolds had resulted in their improved properties. The blend scaffolds of P(3HB-*co*-50mol%4HB)/10% gelatin (50/10) and P(3HB-*co*-82mol%4HB)/7.5% gelatin (82/7.5) at a total weight of

0.395 g had demonstrated better cell proliferation as compared to other blend scaffolds. Additionally, the surface morphology, surface roughness, and water absorption capacity of the 50/10 blend had displayed several exceptional properties, in comparison to those of the 82/7.5 blend. Layer-by-layer nanofibrous scaffolds were prepared into P(3HB-*co*-4HB)/gelatin (PG) and P(3HB-*co*-4HB)/gelatin/P(3HB-*co*-4HB) (PGP), using electrospinning and the average fiber diameter being 250 nm. These two layered nanofibrous scaffolds were prepared in order to study the effect of a different layer on nanofibrous scaffold properties. The layered nanofibrous scaffolds were treated with the vapour of glutaraldehyde. The interaction of glutaraldehyde with gelatin's amino group was reconfirmed using ATR-FTIR which displayed a new peak within the range of 1641-1631 cm^{-1}; indicating the reaction of gelatin with the glutaraldehyde. Also, it was observed that the PGP nanofibrous scaffolds demonstrated stability in the solution for up to 72 hours, which reaffirms its good water-resistant properties whereas the PG nanofibrous scaffolds demonstrated better hydrophilic properties compared to P(3HB-*co*-4HB) and PGP. The results of the MTS assay have shown that the PG and PGP nanofibrous scaffolds show better cell growth compared to the P(3HB-*co*-4HB). In conclusion, the porous scaffold with the composition 50/10 and the nanofibrous scaffold of PGP had displayed suitable properties that are exceptionally applicable in medical fields.

CHAPTER 1: INTRODUCTION

1.1 Research Background

Polyhydroxyalkanoates (PHA) are biodegradable polymers produced by microorganisms under unfavorable growth conditions. These polymers are valuable because they are environmentally friendly, renewable and biocompatible bioplastics (Akiyama et al., 2003; Verlinden et al., 2007). The P(3HB-*co*-4HB) copolymer is the combination resulting in the presence of both short chain length (scl), 3HB and 4HB monomers. The combination of these two different monomers results in a production a series of material with a wide range of mechanical property that can be tailored to specific needs. Interestingly, at 20-40% 4HB, the P(3HB-*co*-4HB) copolymer behaves like elastic rubbers (Martin & Williams, 2003). The poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer is one of biopolymer which has been found promising biomaterial in medical and pharmaceutical applications. This is due to its potential as a biocompatible and absorbable material.

In addition, both the monomeric component of P(3HB) and P(4HB) are found as natural metabolites. P(3HB) release R-3-hydroxybutanoic acid and it is normal metabolite found in human blood as ketone body and is present at concentrations of 3-10 mg per 100 mL blood in a healthy adult. Moreover, the monomeric component of poly(4HB), 4-hydroxybutanoic acid, is also a naturally occurring substance that is widely distributed in the mammalian body, being present in the brain, kidney, heart, liver, lung and muscle (Nelson et al., 1981). The body quickly metabolizes 4HB, and its half-life approximately 27 minutes (Martin & Williams, 2003). 4HB was eliminated from the body primarily by metabolism via the Krebs cycle and ultimately converted to carbon dioxide and water. To ensure the safety of P(4HB), the biological effects of the polymer and devices made it have been extensively evaluated both *in vitro* and *in*

vivo. Besides that, it possesses a relatively good toughness and process properties because of the 4HB linear chain, which reduces the crystallinity and melting temperature compared to other PHAs. In addition, the biodegradation rate of this copolymer is relatively higher *in vivo* and can be regulated by altering the 4HB monomer fraction in copolymer (Martin & Williams, 2003). These property attributes are highly sought for its use in biomedical application especially in drug loading, sutures, and tissue engineering.

Gelatin is derived from parent collagen and possesses characteristics similar to its parent (Meng et al., 2008). Gelatin is extensively used in multiple applications, especially in medical and pharmaceutical fields. This is understandable as it possesses many attractive properties since it is excellent in biodegradable, biocompatibility, non-antigenicity, plasticity and adhesiveness (Kathuria et al., 2009). Besides that, this polymer was proven to be a good film and has oxygen barrier properties (Chiou et al., 2008). In fact, this polymer has shown the ability to support cellular activities including cell attachment and proliferation (Zhang et al., 2005; Zhang et al., 2006).

There are lots of techniques used to manufacture porous scaffolds including solvent casting, salt leaching, freeze-drying and gas foaming (Annabi et al., 2010; Chen et al., 2002). In this study, the solvent casting and freeze-drying techniques were selected to prepare the porous scaffolds. The solvent casting is a versatile technique and is applicable in laboratory scale. This technique is easy, and the formation of the film depends on the mixing of solutions as well as evaporation of solvents. In this study, the solvent casting technique is combined with a freeze-drying technique in order to completely remove the remaining solvent in scaffolds. The freeze-drying technique has been used extensively to create high-porosity scaffolds with the porosity greater than 90% (Sultana & Wang, 2008; Whang et al., 1999).

This technique was proven to produce sponge-type hydrogel and has been utilized in wound dressing. Moreover, the freeze-drying technique has been widely adopted in pharmaceutical and food industries, since it provides stability or rapid solubility in many applications such as drug delivery including site-specific and antibiotic delivery system (Kang et al., 1999). According to Sultana & Wang, (2008) and Jiankang et al., (2009), this technique has been successfully fabricated hydroxyapatite/poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (HA/PHBV) and chitosan-gelatin hybrid porous scaffolds. The scaffolds produced were shown to provide a good environment for cell attachment and proliferation. Subsequently, the scaffolds provided better performance compared to commercial material, Eco-plast on wound healing of Wistar rat (Rahman et al., 2013).

There are many techniques used to produce nanofibers scaffolds such as phase separation, template synthesis, self-assembly, and electrospinning (Nagiah et al., 2013). The electrospinning has gained the interest of researchers because of its promising technique of producing micro or nanofiber (Bhardwaj & Kundu, 2010). The advantage of this technique is the fact that it exhibits high porosity and surface area per volume. Hence, it can be served as a suitable environment for cell attachment and proliferation. Apart from that, the structure of nanofiber has a similar physical dimension to those of natural extracellular matrix (ECM) (Ghasemi-Mobarakeh et al., 2008; Xu et al., 2004). This technique has been successfully fabricated various polymers including natural, synthetic and blend of natural and synthetic polymer. The polymers that has been successfully fabricated into nanofiber were collagen, gelatin, chitosan, hydroxyapatite, silk fibroin, poly(lactic acid) PLA, poly(caprolactone) PCL, poly(lactic-*co*-glycolic acid) PLGA, poly(urethane) PU and the blend materials such as P(3HB-*co*-3HV)/gelatin, P(3HB)/gelatin, P(3HB-*co*-4HB)/collagen, PCL/gelatin

(Huang et al., 2004; Kim et al., 2004; Meng et al., 2008; Nagiah et al., 2013; Yang et al., 2004; Zhang et al., 2005).

This is the first study attempt for the blending of P(3HB-*co*-4HB)/gelatin blend scaffolds that was carried out by using two different techniques, solvent casting and freeze-drying, and electrospinning. The purpose of this study is to produce a high porosity and nanofibrous structured scaffolds. Both techniques of freeze-drying and electrospinning have been reported to produce scaffolds with high porosity and it is advantages for cell attachment and proliferation assay. Based on previous reported, P(3HB-*co*-4HB) blend scaffold was already widely fabricated with another polymer includes collagen, chitosan, cellulose and PLA (Rennukka & Amirul, 2013; Vigneswari et al., 2015; Zhijiang et al., 2012). From the outcome of the findings in the reported paper, it was observed that the blending process have provided a positive effect on physical, mechanical and biological properties. Thus, in this study, the blend material of P(3HB-*co*-4HB)/gelatin was prepared using two different techniques in order to obtain a porous and nanofiber scaffolds. The blend scaffold produced was further characterized for its physical, chemical and biological property.

1.2 Problem Statement

The scarcity of organ donors and tissue for organ substitution that had become necessary due to sickness or accidents causes increasing interest from scientists to develop an alternative method to create new biological substitute. This substitute is required to function well in order to support and regenerate tissue while not causing toxic to host human. It was regenerated from tissue-specific cells and natural or synthetic polymeric matrices (Kang et al., 1999). Subsequently, tissue engineering is one field that fulfils this requirement because it can treat damaged and lost organ (Chen et al., 2002). The aim of this field is to develop a biological substitute that can maintain,

improve or restore the tissue function (Vacanti, 2006). In tissue engineering application, a scaffold is needed as a temporary device for adhesive substrate and to provide physical support for implanted cell especially in the formation of new organs (Chen et al., 2002). The targeted scaffold produced should exhibit various valuable properties such as biocompatible, biodegradable, highly porous, mechanically strong and has pliable property. The importance of these properties to ensure that the scaffold produced functions properly for cell attachment and proliferation purposes (Chen et al., 2002).

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) is a biodegradable and bioresorbable polymer from the PHA group. This copolymer has shown biocompatible with various cell line includes murine fibroblast L929, fibroblast V79, and dermal fibroblast (Chanprateep et al., 2010; Vigneswari, 2015). However, it has a drawback. It possesses hydrophobic property due to the presence of $-CH_3$ side group of P(3HB) and this property could not be adjusted although by varying its 4HB monomer compositions (Rennukka & Amirul, 2013). This property was a disadvantage towards cell performance especially for cell attachment and proliferation of cell line on surface scaffold because of the lack of recognition site (Tallawi et al., 2015). Therefore, it is a disadvantage for further application targeting in medical application, especially for tissue engineering.

The approach to resolve this problem is by hybrid or blending with a natural polymer such as protein, polysaccharide, rubber or carbohydrates. It is one of the best ways to improve the property of the polymer. Natural polymer possesses the high hydrophilic property but weak in the mechanical property (Yu et al., 2006). Hence, by incorporating of P(3HB-*co*-4HB) copolymer with natural polymer, it will balance both polymers properties and resulting to produce novel material with good property in term

of water absorption, hydrophilicity, surface morphology and biocompatible properties. In this study, the P(3HB-*co*-4HB) copolymer was blended with fish gelatin. The purpose of this study is to investigate the effects of incorporation of fish gelatin in the property of blend scaffold.

In this experiment, fish gelatin was selected as opposed to mammalian gelatin in light of the fact that there is no concern on contamination by bovine spongiform encephalitis (BSE) and thus, it is safe to be consumed by ethnic groups that do not consume cow or pig products. Furthermore, fish gelatin can be extracted from low-value byproducts of fishing enterprise such as fish skin, bones, heads and tails which can be presently discarded due to the fact they were considered unsuitable for human consumption. Subsequently, the usage of this product could avoid waste and pollution, it is also economically pleasant (Kristinsson & Rasco, 2000). Gelatin is biological in origin and contains Arg-Gly-Asp (RGD)-like sequences of amino-acids which provides excellent biocompatibility properties for cells attachment and proliferation (Zhang et al., 2005; Zhang et al., 2006). Thus, it will enhance the interaction between cell-scaffolds results in better cell performance.

1.3 Research Objectives:

1) To fabricate and enhance the P(3HB-*co*-4HB)/gelatin blend scaffolds using two different techniques; freeze drying and electrospinning
2) To characterize the P(3HB-*co*-4HB)/gelatin blend scaffolds

CHAPTER 2: LITERATURE REVIEW

2.1 Scaffold

A scaffold is defined as a three-dimensional porous solid biomaterial. Its function is to promote cell-biomaterial interactions, cell adhesion and extracellular matrix deposition. The scaffold enables sufficient transportation of gases, nutrients, and regulatory factors that are vital for cell survival and proliferation (Langer & Tirrell, 2004). In tissue engineering application, a scaffold acts as a temporary device for adhesive substrate as well as to provide physical support for implanted cells especially in the formation of new organs (Chen et al., 2002). The targeted scaffold produced should exhibit various valuable properties such as biocompatible, biodegradable, highly porous, mechanically strong and pliable properties.

These properties are important to ensure that the scaffolds produced can function well especially for cell adhesion, cell growth and cell differentiation. The scaffold can be grouped into two groups: biological and synthetic. The biological scaffolds are derived from human and animal tissues. In contrast, synthetic scaffolds are obtained from various sources of polymers (Dhandayuthapani et al., 2011).

2.2 Biomaterial

A biomaterial is defined as a synthetic structure which acts as scaffolds, matrices or constructs that helps to develop artificial materials to renovate or restore the function of diseased or traumatized tissue in human (Chen et al., 2002; Dhandayuthapani et al., 2011). Biomaterials have been widely used for implant purposes such as sutures, bone plates, heart valves, ligaments and joint replacements (Ramakrishna et al., 2001; Vert, 2005). Initially, the material is designed to resemble the native tissue (Keane & Badylak, 2014). The bone cement, dacron and stainless steel are used because it is relatively inert and tolerable to the body's response.

However, these materials are not biodegradable. This drawback increases the interest of researches to find an alternative resource that could replace and provide similar material property with the earlier materials. The polymer is one of the potential resources as it possesses the biodegradable property desired and has good processability. Both of these properties made the polymer the best candidate to replace the earlier materials (Chen et al., 2002; Keane & Badylak, 2014).

There are two groups of polymeric materials; synthetic and natural (Katz et al., 1999; Temenoff & Mikos, 2000). The synthetic polymers are highly beneficial in biomedical application because of its desirable properties such as its porosity, degradation and mechanical property which can be tailored specifically for each application. Apart from that, this polymer is also cheaper than biologic scaffolds and can be abundantly found in the commercial. Besides that, the synthetic polymers also exhibit a long shelf life (Gunatillake et al., 2006). The most popular synthetic polymer that is widely reported includes poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymer of poly(lactic-*co*-glycolic acid) (PLGA). These polymers are approved for clinical usage and implantable devices by the United State Food and Drug Administration (Ma, 2008). Besides, the PHAs groups from microbial polyesters have been considered for applications in tissue engineering (Chen & Wang, 2002).

2.2.1 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) is a biodegradable polymer which is accumulated by microorganisms under limited nutrients such as nitrogen, oxygen and other essential elements and the presence excess of carbons (Amirul et al., 2008; Reddy et al., 2003). These accumulated polymers are stored inside the cytoplasm of microorganisms in the form of water-insoluble inclusion which serves as intracellular carbon and energy storage (Amirul et al., 2008; Anderson & Dawes, 1990; Doi, 1990;

Sudesh et al., 2000). These polymers are found to be accumulated by microorganisms up to 90% of its cell dry weight (Madison & Huisman, 1999). Interestingly, this polymer property can be altered from stiff to more elastic material to suit the desired applications (Doi, 1990; Sudesh et al., 2000).

The poly(3-hydroxybutyrate) is the first reported PHA group found in *Bacillus megaterium* by Lemoigne, 1926 (Sudesh et al., 2000). There are approximately 150 different constituents of PHA including homopolymer or copolymer that have been identified (Steinbüchel & Lütke-Eversloh, 2003). The diversity of PHAs produced are influenced by several factors including the substrate specificity of the enzyme PHA synthase, the types of carbon precursors used in biosynthesis and the metabolic pathways of the microorganisms (Sudesh & Doi, 2005). These polyesters have been successfully accumulated in nature by more than 300 different Gram-positive and Gram-negative bacteria from 75 different genera. However, only a few bacteria including *Ralstonia eutropha, Alcaligenes latus, Azotobacter vinelandii, Chromobacterium violaceum, Methylotrophs* and *Pseudomonads* accumulate polymers with characteristics that meets the commercial interest (Lee, 1996; Lee et al., 1999).

The presence of PHAs inside the cells can be detected by several staining tests such as Nile blue A and Nile Red. These tests were developed as a simple and highly staining method to directly detect poly(3-hydroxybutyric acid) and other polyhydroxyalkanoic acids (PHA) in growing bacterial colonies. These dyes were added directly in the medium, and the growth of the cells occurred in the presence of the dyes. This allowed an estimation of the presence of PHAs colonies at any time during the growth experiment and the presence of Nile Red or Nile Blue A did not affect the growth of bacteria (Spiekermann et al., 1999). PHA exist as discrete

inclusions that are typically 0.2 to 0.5 μm in diameter, localized in the cell cytoplasm and can be visualized clearly under phase contrast microscope due to their high refractivity (Dawes & Senior, 1973).

The chemical constituents of this polymer can be determined using gas chromatography (GC) and nuclear magnetic resonance analysis (Sudesh et al., 2000). There are several PHAs which are reported commercially and well-studied such as poly(3-hydroxybutyrate) [P(3HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)]. The physical and mechanical properties of these polymers are very similar to the commodity thermoplastics properties (Sudesh & Iwata, 2008).

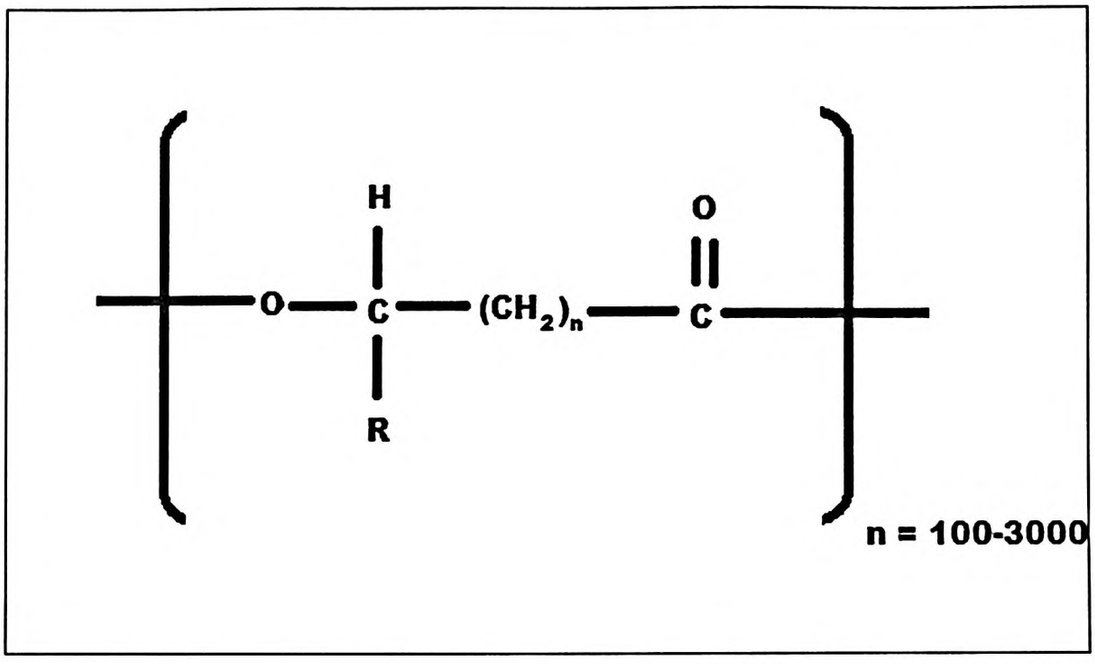
2.2.1(a) Structural properties of PHAs

According to the number of carbon atoms in the monomers, PHAs can be divided into short-chain-length (scl-PHA), medium-chain-length (mcl-PHA) and long-chain-length (lcl-PHA). The short-chain-length (scl-PHA) contains 3-5 carbon atoms in the monomer. Meanwhile, the medium-chain-length (mcl-PHA) contains 6-14 carbon atoms in the monomer (Solaiman et al., 2006; Tsuge, 2002). Moreover, the long-chain-length (lcl-PHA) contains 15 or more carbon atoms in the monomer (Tappel et al., 2014). The example of scl-PHA groups is 3-hydroxybutyrate, 3-hydroxyvalerate and 4-hydroxybutyrate. Scl-PHA has been extensively studied due to its high production (Wang et al., 2016).

The homopolymer of (R)-3-hydroxybutyrate, P(3HB) is a member of scl-PHA and it is the most common type of PHA produced by bacteria in nature. However, is a highly crystalline and the material is stiff, brittle and has poor elastic properties (Tsuge, 2002). This lack of flexibility limits its range of application. Thus, incorporation of

other monomers such as 3-hdroxyvalerate and 4-hydroxybutyrate from scl-PHAs results in a more ductile and tougher material compared to P(3HB) homopolymer (Luzier, 1992). In contrast, the mcl-PHA is regarded as thermoelastomers and rubber (Tsuge, 2002). The example of mcl-PHA groups are 3-hydroxyhexanoate, 3-hydroxy-4-methylvalerate, and 3-hydroxyoctanoate.

A copolyester of 3HB and (R)-3-hexanoate (3HHx), P(3HB-*co*-3HHx), produced by *Aeromonas caviae* was demonstrated to be a flexible material (Doi et al., 1995). Thus, random co-polyesters of 3HB and longer chain (R)-3-hydroxyalkanoates (3HA) seem to have preferable mechanical properties (Tsuge, 2002). The general structure of PHAs is shown in Figure 2.1 (Doi, 1990).



Monomer

n = 1,	R = hydrogen	Poly (3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2,	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
n = 3,	R = methyl	Poly(5-hydroxyvalerate)
	R = ethyl	Poly(5-hydroxyhexanoate)
n = 4,	R = hexyl	Poly(6-hydroxydodecanoate)

Figure 2.1: General structures of polyhydroxyalkanoates (Lee, 1996).

The structure of PHAs produced are influenced by the genetic or physiological strategies of organisms (Steinbüchel & Schlegel, 1991). The pendant group (R) of PHAs produced varies from methyl (C1) to tridecyl (C13) (Verlinden et al., 2007). Usually, the structure of PHAs synthesized inside bacteria is in the R(-) configuration. This is possibly influenced by the stereospecificity of the polymerizing enzyme, PHA synthase. This structure is essential for the biocompatibility and biodegradability of PHAs (Philip et al., 2007; Verlinden et al., 2007; Zinn & Hany, 2005). Apart from that, the length of the side chain and the functional group of PHAs have greatly influenced the physical properties of the polymer such as melting point, glass transition temperature and crystallinity. These properties are crucial in determining the final application of PHAs (Doi, 1990; Verlinden et al., 2007)

2.2.1(b) Poly(3-hydroxybutyrate) [P(3HB)]

P(3HB) is one of the well-studied PHAs group. It has been successfully synthesized by various bacteria in nature. *Ralstonia eutropha*, is a well-known PHA producer and was known to be able to accumulate P(3HB) up to 80% of its cell dry weight from various carbon sources such as sugars, organic acids, and plant oils (Tsuge, 2002). PHB is accumulated inside the bacteria and the pathway of biosynthesized uses pathway I as stated in Figure 2.2. The (R)-3HB pendant group was generated from acetyl-CoAs, commonly found in bacteria. This pathway has been extensively investigated in *Ralstonia eutropha*. The two acetyl-CoA moieties are condensed to yield acetoacetyl-CoA by 3-ketothiolase (PhaA). The product is subsequently condensed to R-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). Only isomers with R configurations are accepted as the substrates of PHA synthase (PhaC). The monomer is polymerized by PHA synthase and resulted in the formation of P(3HB) homopolymer (Anderson & Dawes, 1990).

Pathway I

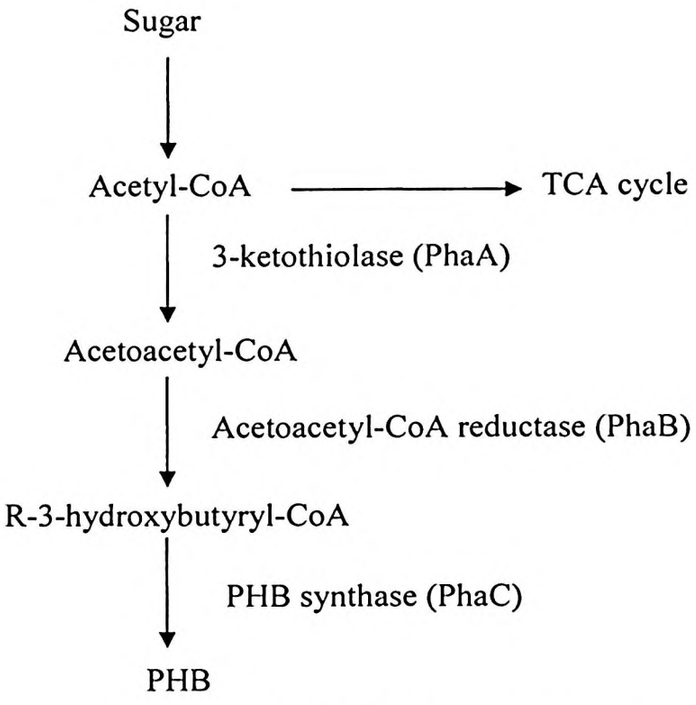


Figure 2.2: Metabolic pathway of P(3HB) (Verlinden et al., 2007).

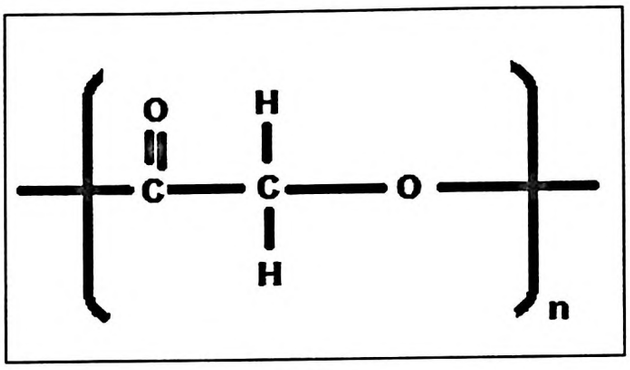
This homopolymer exists inside cells in a fluid or amorphous state. However, the polymer properties changed and became crystalline and stiff, a brittle material after the extraction process (Padermshoke et al., 2005). This polymer has a high molecular weight ranging from 200,000 to 3,000,000 Da. The molecular weight of the polymer depends on the microorganism and the growth conditions during biosynthesis (Ojumu et al., 2004). Furthermore, the number-average molecular weight (M_n) of P(3HB) homopolymer is found to be 20 MDa, a result obtained from biosynthesis using a recombinant strain of *E. coli* (Kusaka et al., 1999). Apart from that, this homopolymer possesses excellent biocompatibility as it is non-toxic, support cell proliferation and is the degradation product of P(3HB) polymer. It had resulted in the production of 3-hydroxybutyrate that is known as a natural metabolite in animal host that is associated to human ketone body formation (Brigham & Sinskey, 2012; Sudesh et al., 2000). P(3HB) has been identified in blood serum complexed with low-density lipoproteins

and with the carrier of protein albumin (Williams & Martin, 2005). However, the drawback of this homopolymer is the melting temperature (around 170 °C) seems to be too close to its decomposition temperature and consequently restricted its application (Madison & Huisman, 1999).

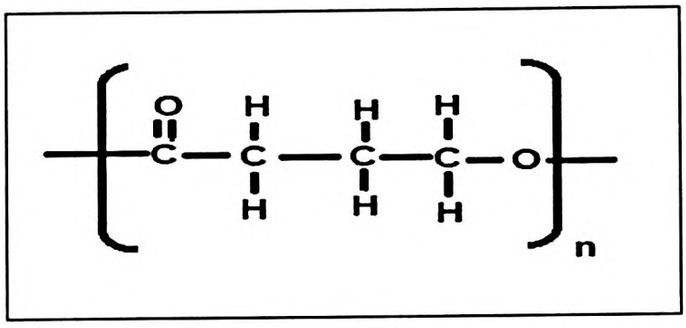
2.2.1(c) Poly(4-hydroxybutyrate) [P(4HB)]

The P(4HB) homopolymer is produced naturally by microorganisms and was established as new absorbable biomaterials for medical application (Doi, 1990; Madison & Huisman, 1999; Martin & Williams, 2003). This polymer was successfully synthesized using recombinant *Escherichia coli* K12 via the fermentation process (Williams et al., 2013). The chemical structure of P(4HB) homopolymer resembles the existing synthetic resorbable polymers such as polyglycolide (PGA) and poly-ε-caprolactone (PCL) (Figure 2.3). This homopolymer exhibits a semi-crystalline property with a melting point of approximately 60 °C and glass transition temperature (T_g) of -51 °C (Martin & Williams, 2003; Williams et al., 2013). Besides, it is also a strong pliable thermoplastic that has more flexibility property compared to other synthetic absorbable polymers including polyglycolide (PGA) and poly-L-lactide (PLLA), as shown in Table 2.1 (Martin & Williams, 2003).

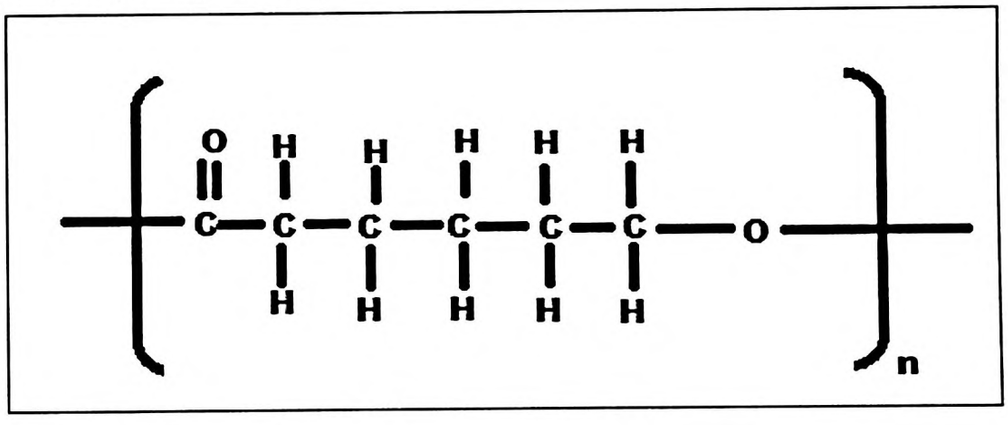
This material is sterilized using cold-ethylene or gamma radiation prior to use in the medical field. This is to ensure the material is safe to be used for the *in vitro* or implantation. The sterilization using cold-ethylene yields traces of residuals. In contrast, the gamma radiation gives side effects such as reduction in molecular weight (M_w) and strength of polymer (Williams et al., 2013). This homopolymer is the first PHAs material approved by the FDA for the clinical application of absorbable suture (TephaFLEX). This material is also applicable in tissue engineering and drug delivery due to its strong, flexible, biocompatible properties. It is also well tolerated especially



Polyglycolide



Poly(4-hydroxybutyrate)



Polycaprolactone

Figure 2.3: Chemical structure of PGA, P(4HB), and PCL (Williams et al., 2013)

Table 2.1: Comparison of properties of P(4HB) with other absorbable thermoplastic polyesters (Martin & Williams, 2003).

	T_m (°C)	T_g (°C)	Tensile strength (MPa)	Tensile modulus (MPa)	Elongation at break (%)	Absorption rate
PGA	225	35	70	6900	<3	6 weeks
PLLA	175	65	28-50	1200-2700	6	1.5-5 years
DL-PLA	Amorphous	50-53	29-35	1900-2400	6	3 months
P(3HB)	180	1	36	2500	3	2 years
PCL	57	-62	16	400	80	2 years
P(4HB)	60	-51	50	70	1000	8-52 weeks

Abbreviations are PGA, poly(glycolic acid), PLLA, poly(L-Lactic acid), DL-PLA, a copolymer of D-lactide and L-lactide; P(3HB), poly(3-hydroxybutyrate); PCL, polycaprolactone.

in vivo study. On the other hand, the monomeric component of P(4HB), 4-hydroxybutyric is a naturally occurring substance that is widely distributed in the mammalian body, present in the brain, kidney, heart, liver, lung and muscle (Nelson et al., 1981). This 4-hydroxy acid has been used as an intravenous agent for the induction of anesthesia and for long-term sedation (Williams & Martin, 2005). Besides that, this monomer is eliminated from the body by metabolism via the Krebs cycle where it is converted into carbon dioxide and water (Williams et al., 2013).

The level of endotoxin content of this material was evaluated and found to meet the standards, which is not exceeding 20 US Pharmacopeia (USP) endotoxin units per device (Tepha, 1995). This test is crucial for materials that were synthesized from Gram-negative microorganism because the endotoxin is found as an integral

component on the outer cell surface. High endotoxin content in the material is capable of inducing fever to humans (Martin & Williams, 2003). Interestingly, the degradation rate of P(4HB) homopolymer is comparable to the other biopolymers. Nonetheless, P(4HB) possesses a slower degradation rate compared to the PGA, but faster than the PLLA, PCL and P(3HB) in a subcutaneous environment (Table 2.1).

2.2.1(d) Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)], is a copolymer, combining both 3HB and 4HB monomers that exhibits useful property relative to other PHAs. The combination of both monomers is advantageous because both are comprised of relatively good biomaterial properties. The structure of this copolymer is shown in Figure 2.4. This copolymer was successfully synthesized by *Ralstonia eutropha* and first identified in 1988 (Doi et al., 1988). Five wild-type bacteria have been reported to produce P(3HB-*co*-4HB) copolymer which includes *Ralstonia eutropha*, *Alcaligenes latus*, *Comamonas acidovorans*, *C. testosteronii* and *Hydrogenphaga pseudoflava* (Kang et al., 1995; Kim et al., 2005; Mitomo et al., 2001; Renner et al., 1996). Among these microorganisms, *R. eutropha* has been widely reported to have the ability to synthesize P(3HB-*co*-4HB) copolymer with various 4HB monomer compositions ranging from 0-100 mol%. This copolymer can be produced by microorganism using various carbon precursors, either sole or mixed carbon substrate. The sole carbon precursors of 4HB such as 4-hydroxybutyric acid, 1,4-butanediol and γ-butyrolactone are commonly used to produce the copolymer. On the other hand, the usage of mixed carbon substrate of 4HB precursors including the γ-butyrolactone + 1,6-hexanediol, 1,4-butanediol + 1,6-hexanediol, and precursors including the γ-butyrolactone + 1,4-butanediol in the fermentation process via one-stage cultivation by *Cupriavidus* sp. USMAA1020 are successfully produced by

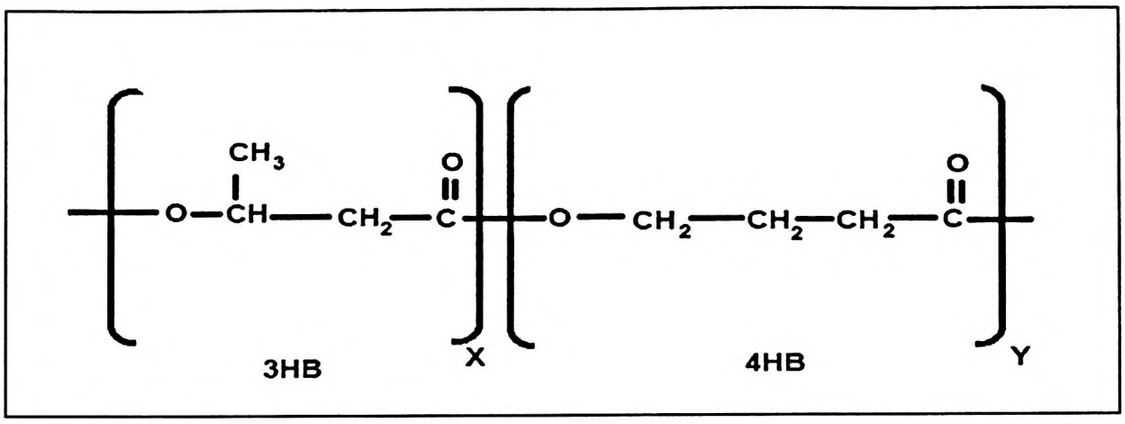


Figure 2.4: Chemical structure of P(3HB-*co*-4HB) copolymer (Chee et al., 2008)

P(3HB-*co*-4HB) copolymer with various 4HB monomer compositions ranging from 7 mol% to 70 mol% (Huong et al., 2014).

For the biosynthesis of P(3HB-*co*-4HB) copolymer, the metabolic pathway is shown in Figure 2.5. The biosynthesis pathway of the copolymer is influenced by the carbon precursors supplied during the production process. The carbon precursor is normally converted into 4-hydroxybutyryl-CoA before being polymerized by PhaC into the 4HB polymer. For γ-butyrolactone, it is hydrolytically cleaved into 4HB by esterase or lactonase and metabolized into 4-hydroxybutyryl-CoA (4HB-CoA). Meanwhile, for ω-alkanediols such as 1,4-butanediol, 1,6-hexanediol and 1,8-octanediol, these substrates are oxidized via enzymatic reactions including β-oxidation to form 4-hydroxybutyric acid which also leads to the formation of 3HB intermediate, 3-hydroxybutyryl-CoA. The generation of 3HB polymer leads to the accumulation of P(3HB-*co*-4HB) copolymer (Steinbüchel & Lütke-Eversloh, 2003).

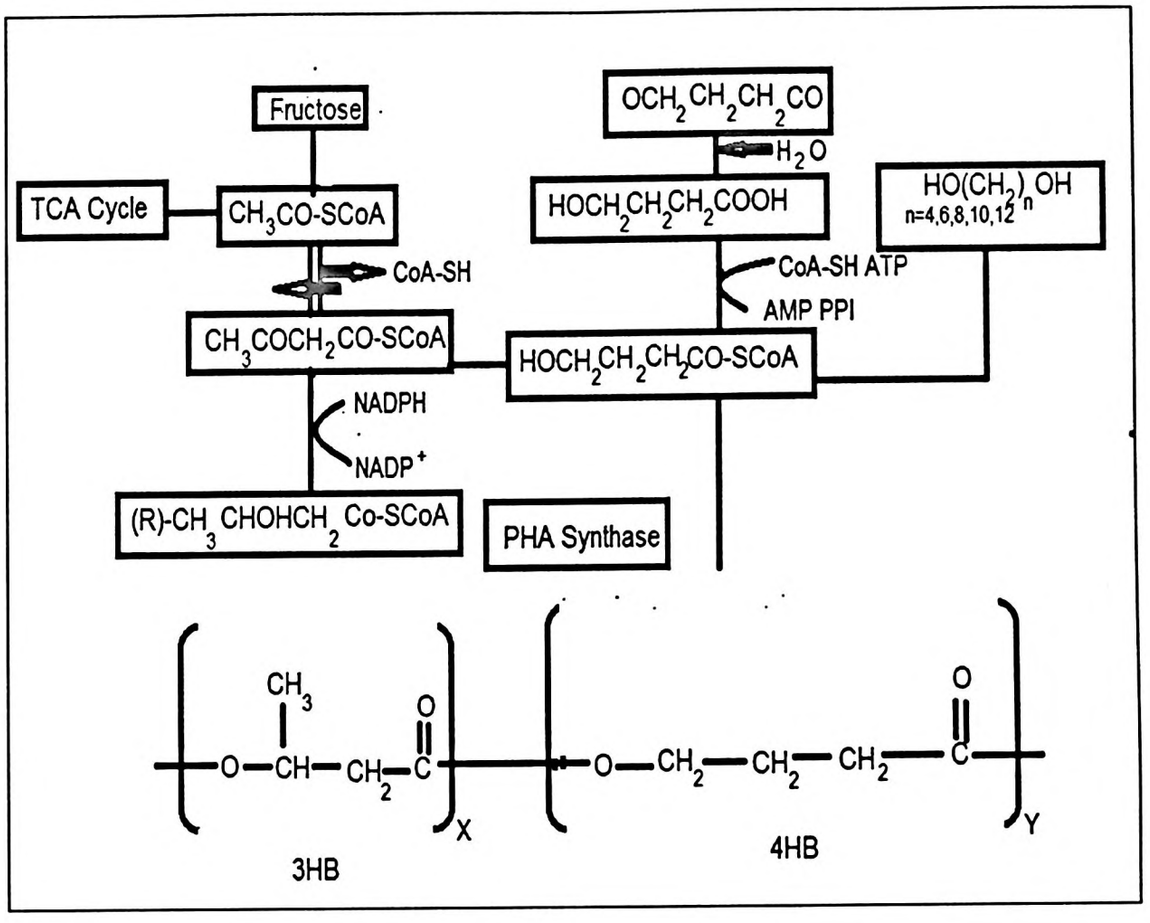


Figure 2.5: The biosynthesis pathway of P(3HB-*co*-4HB) in *A. eutrophus* (Saito et al., 1996).

The incorporation of the 4HB unit into P(3HB) homopolymer appears to improve the physical properties of this polymer from a highly crystalline material to a strong elastomeric rubber-like material (Doi, 1990; Saito & Doi, 1994). For example; the incorporation of 16 mol% 4HB into P(3HB) had increased the extension to break of the polymer from 5% up to 444% and tensile strength, 26 MPa, respectively (Tsuge, 2002). Moreover, the T_m and T_g of this copolymer decreases concurrently with the increase of 4HB monomer compositions (Saito & Doi, 1994). Apart from that, the P(3HB-*co*-4HB) copolymer possesses desirable mechanical properties which are applicable in medical and pharmaceutical fields (Martin & Williams, 2003). Besides, it is found that this polyester exhibits therapeutic property as both 3-hydroxybutyrate and 4-hydroxybutyrate are natural metabolites present in the human body (Sudesh et al., 2000). In fact, the degradation rate of this copolymer *in vivo* is relatively higher than the other PHAs group. Interestingly, the degradation rate of this copolymers can be manipulated by varying the 4HB monomer compositions (Martin & Williams, 2003).

2.2.1(e) Biocompatibility of PHAs

The concept of tissue engineering is built based on the interaction between the cell-material system. The biocompatibility of polymers is needed for cell attachment, proliferation, migration and differentiation. All PHAs is known to possess biocompatibility and several studies conducted on P(3HB) had yielded good biocompatible properties with various cell lines including osteoblast, epithelial cell and chondrocytes (Nebe et al., 2001; Rivard et al., 1996). The biocompatibility of materials was further observed on implant material by studying the changes in the structure of cellular molecules during cell adhesion on P(3HB) film (Nebe et al., 2001).

The P(3HB-*co*-4HB) copolymer is another group of PHAs that has the potential to be applied as a biomaterial. The P(3HB-*co*-4HB) copolymer with various 4HB monomer compositions ranging from 23 mol% to 75 mol%, was discovered to be able to support the growth and proliferation of cell L929 on its film (Vigneswari et al., 2009). Apart from that, it could be observed that the P(3HB-*co*-4HB) film with monomer compositions of 5, 24 and 38 mol%, were evaluated for the dermal application using fibroblast cells, L929 and human dermal. The proliferation of human dermal fibroblast cells on films was observed to increase proportionally with an increase in 4HB monomer compositions (Chanprateep et al., 2010). Based on the findings, different cells prefer distinct types of surface for cell attachment purposes. The P(3HB-*co*-4HB) copolymer exhibits different surface morphology depending on its molar fraction (Chee et al., 2008). *In vitro* study is the initial step in determining the biocompatibility of material (Siew et al., 2007).

2.2.2 Gelatin

Gelatin is derived from native collagens, which are found abundantly in skin, tendon and bone of animals. It is a water-soluble protein composed of a variety of amino acids which are connected via amide bonds (Schrieber & Gareis, 2007). Gelatin is found abundantly from sources such as pig's skin (46%), bovine hide (29.4%) and pork and cattle bones (23.1%). Moreover, fish gelatine is found to be around 1.5% (Wasswa et al., 2007). Fish gelatin was observed to be a major advantage as replacement sources because of certain religious sensitivity (Judaism, Hinduism and Islam) and the risk of outbreaks of disease, Bovine Spongiform Encephalopathy (BSE) (Chiou et al., 2008).

Gelatin is a promising material with a lot of advantages including cost efficiency, hydrogel characteristics, biodegradability, non-toxicity and

biocompatibility (Li et al., 2006). This polymer has proven itself as a good film-forming and had shown good oxygen barrier properties (Chiou et al., 2008). Apart from that, its biological origin containing the Arg-Gly-Asp (RGD)-like sequences of amino-acids are providing excellent biocompatibility properties for cell attachment and proliferation purposes (Zhang et al., 2005; Zhang et al., 2006). This polymer has been widely used in the pharmaceutical and medical fields (Gu et al., 2009).

The physical and chemical properties of gelatin depends on factors such as sources, animal age, collagen type and manufacturing method (Gomes et al., 2013; Gómez-Guillén et al., 2009). Cold-water fish gelatins, such as those extracted from pollock and salmon, have very low gelation and melting temperatures compared to mammalian and warm-water fish gelatins. This is due to cold-water fishes having lower concentrations of proline and hydroxyproline than any other species (Haug et al., 2004). The amino acid compositions of mammalian gelatins are remarkably constant compared to those of different species of fish (Avena-Bustillos et al., 2006). Fish gelatin shows a wider variation in composition. Their hydroxyproline and proline contents are lower than that of mammalian gelatin and this is compensated by having a higher concentration of serine and threonine. Consequently, cold-water fish gelatin behaves as a viscous liquid at room temperature, which limits their use in many applications (Avena-Bustillos et al., 2006). However, fish gelatin has an added advantage where it exhibits lower immunogenicity compared to mammalian gelatin (An et al., 2010; Chiou et al., 2008). In addition, the fish gelatin had shown their excellent film-forming properties with a potential of high barrier efficiency that could be applied for edible films and coatings (Avena-Bustillos et al., 2006).

The blend study with gelatin/PLLA, PCL/gelatin or P(3HB)/gelatin has successfully been fabricated (An et al., 2010; Ghasemi-Mobarakeh et al., 2008; Nagiah

et al., 2013). The cell study had shown better cell growth and proliferation on the blended composite (An et al., 2010). This blend had already proven itself applicable in dermal reconstruction, nerve tissue engineering and others (An et al., 2010; Kim et al., 2008).

2.3 Polymer blending

Polymer blending is a mixture of more than one existing polymer for the purpose of fabricating a new material with excellent and wide range properties. This property could be achieved by manipulating the blend ratios during the preparation steps. This technique has an advantage because it is cost efficient and save time compared to synthesizing new polymers or developing new polymerization routes (Koning et al., 1998). In most studies, the blending process is carried out to enhance the properties of polymer especially natural polymer, since most of them exhibited weak mechanical and temperature properties. The blending of natural with synthetic polymer will stabilize their properties. The selection of natural polymer is the key to determining its properties and can therefore perform diverse functions depending on its native origin (Yu et al., 2006).

Most natural polymers are hydrophilic materials since they contain either hydroxyl or polar groups. On the other hand, most synthetic biodegradable polymers, especially aliphatic polyesters are hydrophobic or sensitive to moisture. Blending these two kinds of polymers together is of significant interest since it could lead to the development of a new range of biodegradable polymeric materials (Yu et al., 2006).

Based on a previous report, the P(3HB-*co*-4HB)/chitosan had demonstrated to significantly improved its properties with the increase of chitosan content. The P(3HB-*co*-4HB) copolymer is well-known to exhibit hydrophobic property in nature. It was also observed that the increase in 4HB monomer compositions did not affect its