CHARACTERIZATION OF A HIGHLY ACTIVE POLYHYDROXYALKANOATE SYNTHASE

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CHARACTERIZATION OF A HIGHLY ACTIVE POLYHYDROXYALKANOATE SYNTHASE

by

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL NAME
PhaA	*β*-ketothiolase
3HB	3-hydroxybutyrate
(*R*)-3HB-CoA	3-hydroxybutyryl-CoA
3HHx	3-hydroxyhexanoate
(*R*)-3HHx-CoA	3-hydroxyhexanoyl-CoA
3HV	3-hydroxyvalerate
(*R*)-3HV-CoA	3-hydroxyvaleryl-CoA
4HB	4-hydroxybutyrate
X-Gal	5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
NH_4OH	Ammonium hydroxide
BSA	Bovine serum albumin
CME	Caprylic acid methyl ester
^{13}C	Carbon-13
C	Carbon atom
CoA	Coenzyme-A
CPKO	Crude palm kernel oil
CPO	Crude palm oil
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
$CDCl_3$	Deuterated chloroform
DO	Dissolved oxygen

DCW	Dry Cell Weight
EtBr	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
GPC	Gel permeation chromatography
T_g	Glass transition temperature
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
HA-CoA	Hydroxyacyl-CoA
HA	Hydroxyalkanoate
IPTG	Isopropyl-β-D-thiogalactopyranoside
$C_{12:0}$	Lauric acid
LB	Luria Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MCL	Medium chain length
T_m	Melting temperature
CH_2	Methylene group
MM	Mineral medium
M_w	Molecular weight
$C_{14:0}$	Myristic acid
PhaB	NADPH-dependent acetoacetyl-CoA dehydrogenase
NMR	Nuclear magnetic resonance
M_n	Number-average molecular weight
NR	Nutrient rich

OD	Optical density
PAO	Palm acid oil
PO	Palm olein
PhaZ	PHA depolymerase
PhaC	PHA synthase
PhaP	Phasin
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HV-*co*-3HHp)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyheptanoate)
P(3HB-*co*-3HV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
M_w/ M_n	Polydispersity index
PHA	Polyhydroxyalkanoate
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
KOH	Potassium hydroxide
^{1}H	Proton
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PhaR	Regulator protein of the phasin expression
PhaJ	(*R*)-specific enoyl-CoA hydratase
SCL	Short chain length
AOT	Sodium bis(2-ethylhexyl)sulfosuccinate
NaCl	Sodium chloride

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
PhaE	Subunit of PHA synthase
H_2SO_4	Sulfuric acid
TEM	Transmission electron microscope
TCA	Tricarboxylic acid
TAE	Tris-acetate-EDTA
UV	Ultraviolet
$CO(NH_2)_2$	Urea

LIST OF UNITS AND SYMBOLS

UNITS AND SYMBOLS	FULL NAME
β	Beta
cm	Centimeter
Da	Dalton
°C	Degree Celcius
wt%	Dry weight percent
g	Gram
ΔH_m	Heat of fusion
h	Hour
J	Joule
kbp	Kilo base pairs
kDa	Kilodalton
L	Liter
MHz	Megahertz
μg	Microgram
μL	Microliter
μM	Micromolar
μmol	Micromole
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar

min	Minute
M	Molar
mol%	Mole percent
nm	Nanometer
N	Normality
ppm	Parts per million
%	Percentage
psi	Pounds per square inch
rpm	Revolutions per minute
× *g*	Times gravity
V	Volt
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

PENCIRIAN POLIHIDROKSIALKANOAT SINTASE

YANG SANGAT AKTIF

ABSTRAK

Polihidroksialkanoat (PHA) sintase dari *Chromobacterium* sp. USM2 ($PhaC_{Cs)}$ pencilan tempatan mempamerkan aktiviti pempolimeran yang tinggi dan pengkhususan substrat *in vivo* yang luas dengan keutamaan untuk monomer kepanjangan rantai pendek (SCL) [3-hidroksibutirat (3HB) dan 3-hidroksivalerat (3HV)] dan monomer kepanjangan rantai sederhana (MCL) [3-hidroksiheksanoat (3HHx)]. Untuk pencirian sintase secara lebih terperinci, $PhaC_{Cs}$ yang mempunyai tag Strep2 dibina dalam kajian ini untuk ekspresi dan purifikasi dari *Escherichia coli*. Ujian enzim *in vitro* telah menunjukkan aktiviti sebanyak 253 ± 13 U/mg untuk pempolimeran 3-hidroksibutiril-koenzim A (3HB-CoA), yang lebih kurang 5 kali ganda lebih tinggi daripada aktiviti yang ditunjukkan oleh stren contoh dalam penghasilan PHA (39 ± 5 U/mg). Aktiviti pempolimeran 3-hidroksivaleril-koenzim A adalah dua kali ganda lebih tinggi berbanding dengan aktiviti pempolimeran 3HB-CoA, dan aktiviti pempolimeran 3-hidroksiheksanoil menandingi aktiviti yang ditunjukkan oleh sintase SCL-MCL dari *Aeromonas caviae*. Penemuan ini telah mendorong kajian yang lebih mendalam dan aplikasi sintase yang beraktiviti tinggi ini untuk penghasilan PHA *in vivo*. Gen yang mengkod sintase PHA dari *Chromobacterium* sp. USM2 ($phaC_{Cs}$) telah digunakan untuk menggantikan gen sintase PHA asal dalam *C. necator* jenis liar melalui rekombinasi homolog. Stren hasilan ini menunjukkan peningkatan dalam penghasilan poli(3-hidroksibutirat-*co*-3-hidroksiheksanoat) fleksibel dan pertumbuhan yang lebih baik daripada minyak kelapa sawit mentah. Pelbagai strategi seperti pengubahan parameter kultur,

penambahan sebatian prekursor atau perencat dan manipulasi laluan biosintetik telah berjaya untuk meningkatkan fraksi monomer 3HHx yang menambahkan lagi fleksibiliti kopolimer. Faktor-faktor yang mempengaruhi fraksi 3HHx dan ciri-ciri polimer seperti aktiviti sintase, jisim molekul dan morfologi granul telah dikaji secara selari. Sintesis poli(3-hidroksibutirat-*co*-3-hidroksivalerat-*co*-3-hidroksiheksanoat) dengan komposisi monomer yang pelbagai telah menghasilkan bahan yang berciri baru. Mutasi untuk menambahbaikkan spesifisiti terhadap komonomer boleh meningkatkan lagi versatiliti enzim yang wujud secara semulajadi ini. Mutagenesis titik penepuan di posisi 479 dalam $PhaC_{Cs}$ telah dilakukan dan kesan daripada penggantian asid amino telah diuji menerusi ekspresi dalam *E. coli* LS5218 untuk biosintesis PHA daripada asid dodekanoik. Peningkatan dalam kandungan 3HHx dan/atau akumulasi PHA diperhatikan untuk beberapa mutan, mencapai tahap maksima yang lebih kurang 4 kali ganda dan 1.6 kali ganda, masing-masing, melebihi sintase jenis liar, menekankan lagi kepentingan posisi mutasi ini dalam mengubah suai ciri-ciri $PhaC_{Cs}$. Pemerhatian ini boleh dihubungkaitkan dengan penambahbaikkan aktiviti dan peningkatan dalam afiniti $PhaC_{Cs}$ untuk monomer 3HHx. Konsistensi dalam afiniti sintase, sama ada jenis liar atau mutan, terhadap monomer 3HB dan 3HV atau 3HHx secara jelasnya menekankan batasan di antara monomer SCL dan MCL, mengukuhkan lagi dasar untuk pengkelasan PHA sintase. Secara keseluruhannya, keputusan kajian ini mengusulkan konsistensi dalam spesifisiti substrat $PhaC_{Cs}$ *in vivo* dan *in vitro*.

CHARACTERIZATION OF A HIGHLY ACTIVE POLYHYDROXYALKANOATE SYNTHASE

ABSTRACT

Polyhydroxyalkanoate (PHA) synthase from a locally isolated *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) exhibited superior polymerizing ability and broad *in vivo* substrate specificity with preferences for short chain length (SCL) [3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV)] and medium chain length (MCL) [3-hydroxyhexanoate (3HHx)] monomers. For further characterization of the synthase, a Strep2-tagged $PhaC_{Cs}$ for expression in and purification from *Escherichia coli*, was constructed in this study. *In vitro* enzymatic assay revealed an activity of 253 ± 13 U/mg for polymerization of 3-hydroxybutyryl-coenzyme A (3HB-CoA), which was approximately fivefold higher than that of model PHA-producing strain *Cupriavidus necator* (39 ± 5 U/mg). Its activity for polymerization of 3-hydroxyvaleryl-coenzyme A was twice as great as that for 3HB-CoA, while corresponding activity for 3-hexanoyl-coenzyme A polymerization rivaled that of another SCL-MCL synthase, from *Aeromonas caviae*. This discovery prompted further characterization studies and application of the highly active synthase for *in vivo* PHA production. Here, the gene encoding the PHA synthase from *Chromobacterium* sp. USM2 ($phaC_{Cs}$) was used to replace the native PHA synthase gene in wild type *C. necator* by homologous recombination. The resultant strain showed improved productivity of flexible poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from crude palm kernel oil with concomitant good growth. Various approaches such as alteration of culture parameters, addition of precursor or inhibitor compounds and manipulation of biosynthetic pathway successfully

increased the 3HHx monomer fraction, further enhancing flexibility of the copolymer. The factors affecting 3HHx fraction and those governing polymer properties, such as synthase activity, molecular weight and granule morphology, were studied in parallel. Successful synthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) with diverse monomeric composition yielded materials with novel properties. Mutations to enhance specificity of this naturally occurring enzyme towards comonomer can further improve its versatility. Hence, saturation point mutagenesis at position 479 in $PhaC_{Cs}$ was carried out and the effect of the amino acid substitutions was examined by expression in *E. coli* LS5218 for PHA biosynthesis from dodecanoic acid. Increment in 3HHx content and/or PHA accumulation was observed of some mutant synthases, up to a maximum of approximately fourfold and 1.6-fold respectively, more than the wild type synthase, highlighting the significance of this mutation point in altering the properties of $PhaC_{Cs}$. These observations could be correlated with improved activity and increased preference of $PhaC_{Cs}$ for 3HHx monomers. The consistency observed in the preference of synthases, wild-type and mutants alike, for either 3HB and 3HV or 3HHx monomer(s) clearly emphasizes the boundary between SCL and MCL monomers, substantiating the very basis on which PHA synthases are classified. Overall, results suggest consistency in *in vivo* and *in vitro* substrate specificities of $PhaC_{Cs}$.

1.0 INTRODUCTION

Biological organisms have evolved diverse systems for storing essential nutrients, such as carbon, nitrogen, and phosphorous. This storage frequently entails the accumulation of polymers, which can be depolymerized when the monomers are needed for synthesis of other metabolites or for energy generation. These polymers often form insoluble inclusions, which are beneficial because they do not influence reactions involving soluble substrates, and because the polymers do not contribute to the osmotic potential of the cell in which they are stored. Carbon storage molecules, in particular, are more widespread and have greater industrial importance. One such example is polyhydroxyalkanoates (PHAs).

PHAs are polyoxoesters synthesized by a wide range of bacteria as intracellular storage materials (Anderson and Dawes, 1990; Doi, 1990). The unique properties of PHA such as its thermoplastic capabilities and inherent degradability have made it a worthwhile alternative to conventional petrochemical plastics, overcoming the setbacks of this innovation in terms of the lack of degradability. PHAs vary substantially in their composition, resulting in a huge diversity of material properties (Steinbüchel and Lütke-Eversloh, 2003). A homopolymer of 3-hydroxybutyrate [P(3HB)] has a high degree of crystallinity, giving rise to material that is strong but very stiff and brittle, which limits its commercial potential. PHA copolymers have more favorable properties, and a well-studied example is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], which is tougher and more flexible than P(3HB). A copolymer of 3-hydroxybutyrate (3HB) [C4] and 3-hydroxyhexanoate (3HHx) [C6] as the minor constituent (~ 5 mol%) exhibited improved physical properties and thermal processability (Doi *et al.*, 1995; Loo *et al.*, 2005; Matsusaki *et al.*, 2000).

In vivo synthesis of a copolymer with such desirable properties would require a PHA synthase (PhaC) that is able to polymerize short chain length (C3 – C5, SCL-PHA) or medium chain length (C6 – C14, MCL-PHA) monomers produced within the cell. PHA synthases are divided into four classes based on their primary amino acid sequences and substrate specificity (Potter and Steinbüchel, 2005; Rehm, 2003). For class I and class II enzymes, the active synthases are homodimers composed of a single subunit, while class III and class IV synthases require two subunits for full activity. Class I, III and IV synthases produce SCL-PHA, while class II synthases prefer MCL substrates. Some synthases are difficult to classify with this system as intriguingly, they are able to make SCL-MCL copolymers, such as the chosen subject for this study. The synthase in question, designated $PhaC_{Cs}$, originated from the violacein-producing *Chromobacterium* sp. USM2 which was isolated from a waterfall in Langkawi, Malaysia (Bhubalan *et al*., 2010b). Aside from this, only a few other bacteria such as *Aeromonas caviae* (Doi *et al.*, 1995; Kobayashi *et al.*, 1994), *Aeromonas hydrophila* (Chen *et al.*, 2001; Lee *et al.*, 1999) and *Rhodococcus ruber* (Haywood *et al.*, 1991) have been reported to possess synthases that exhibit specificity towards both SCL- and MCL-PHA monomers.

While the broad substrate preference of these SCL-MCL synthases enables production of the practical copolymer, $PhaC_{Cs}$ exhibited superior polymerization activity that provides the added advantage of increased production efficiency (Bhubalan *et al*., 2011). Sufficient understanding of enzyme properties is imperative for application of this versatile enzyme, hence, $PhaC_{Cs}$ was purified in this study for further characterization and its specific activities for polymerization of various substrates were examined *in vitro*.

Besides substrate specificity of the PHA synthase, another important criterion for the design of PHAs using *in vivo* systems is the metabolic potential of the production organism to provide the required precursors. The well-studied *Cupriavidus necator* H16 is a promising candidate with the suitable metabolic pathway for provision of C4 – C6 substrates (Byrom, 1992; Pohlmann *et al*., 2006). This model PHA producer is able to accumulate large quantities of polymer when grown in nutrient limited conditions and in addition, preferentially utilizes plant oils as substrate (Fukui and Doi, 1998). Renewable feedstocks such as plant-based oils are ideal for cost-efficient mass production due to their high carbon content (Akiyama *et al*., 2003) and because metabolism of these compounds can influence monomer composition of the resultant polymer (Bhubalan *et al*., 2010b). *C. necator* H16, however, only produces SCL-PHA and is thus limited as an industrial PHA-producing organism.

In this study, $PhaC_{Cs}$ was used to create a novel recombinant, with *C. necator* H16 as parental strain, to overcome limitations of its native and transformant strains in terms of monomer supply, PHA accumulation and growth ability (Bhubalan *et al*., 2010a,b). The recombinant strain, designated $H16C_{Cs}$, was used to synthesize P(3HB-*co*-3HHx) from crude palm kernel oil as sole carbon substrate followed by preliminary up-scaling of copolymer production in a 10 L fermenter. As variation in 3HHx content impacts change in copolymer properties (Asrar *et al*., 2002), the factors affecting 3HHx fraction was studied. Improvement of 3HHx monomer fraction was attempted by alteration of culture parameters and biosynthetic pathway manipulation. Given the high preference of $PhaC_{Cs}$ for 3-hydroxyvalerate (3HV) monomers and the ability to vary this content (Bhubalan *et al*., 2010a), $H16C_{Cs}$ was evaluated for potential production of a terpolymer comprising 3HB, 3HV and 3HHx

with new monomeric composition. Comparison of $PhaC_{Cs}$ in various physiological environments was made to acquire further insights on the behavior of this synthase and to gain a more profound understanding on the various aspects of PHA biosynthesis in general.

Ultimately, modification of the PHA synthase by means of evolutionary engineering is the best approach to attain polymers with novel composition and properties. Extensive efforts in this direction have yielded various mutant synthases with dramatic improvements in their activities for polymerization as well as novel substrate-binding properties (Nomura and Taguchi, 2007; Taguchi and Doi, 2004). As $PhaC_{Cs}$ demonstrated unusually high polymerization activity and broader substrate specificity as compared to other synthases of the same class, mutations to enhance the preference of this naturally occurring synthase towards comonomer can further improve its versatility. Multiple sequence alignment of $PhaC_{Cs}$ with other comprehensively studied synthases in this area revealed a highly conserved alanine residue among class I synthases, located at position 479 in $PhaC_{Cs}$. Functional implications of amino acid substitutions at the position based on previous studies include alterations in substrate specificity and in certain cases, variations in molecular weight of the resultant polymer were observed (Takase *et al*., 2003; Tsuge *et al*., 2004b; Tsuge *et al*., 2007a). This position was therefore selected for saturation mutagenesis of the $PhaC_{Cs}$-encoding gene ($phaC_{Cs}$) in this study, and the mutagenized fragments were introduced into an *Escherichia coli* mutant, individually, for evaluation of P(3HB-*co*-3HHx) production from dodecanoic acid. Aside from the acquisition of $PhaC_{Cs}$ mutants with novel properties, the effects of these mutations will enable the identification of factors that are essential to the enzyme's function and the interaction between these factors can be examined.

Therefore, the main objective of this study was to characterize the PHA synthase from *Chromobacterium* sp. USM2 in an effort to attain a more profound understanding on its substrate preference and to determine its specific activities for the polymerization of various substrates. With increased knowledge on $PhaC_{Cs}$, this study also aimed to further improve this synthase in terms of its ability to incorporate 3HHx by various approaches in parallel to studying the factors influencing 3HHx incorporation. The means described above were employed to achieve these objectives.

2.0 LITERATURE REVIEW

2.1 Polyhydroxyalkanoate (PHA): An overview

In living organisms, polymers are synthesized to fulfil biological functions for survival. These storage polymers are synthesized in a non template-dependent manner, as opposed to deoxyribonucleic acid, ribonucleic acid, and proteins, whose synthesis is directed by information encoded in other biopolymers (Stubbe *et al.*, 2005). Nitrogen can be stored as cyanophycin (Mooibroek *et al*., 2007), while phosphorous can be stored in the form of polyphosphate (Kulaev and Kulakovskaya, 2000). These compounds are of significant academic interest, particularly carbon storage molecules, as they have greater industrial importance.

Bacteria have been found to store carbon in the form of glycogen (Preiss, 1984), triacylglycerols (Alvarez and Steinbüchel, 2002), and polyhydroxyalkanoates (PHAs) (Anderson and Dawes, 1990). It has been shown that in environments with fluctuating carbon levels, PHA producers thrive better than rival species (Johnson *et al.*, 2009). PHAs are polymers of hydroxyalkanoates, which are accumulated as an intracellular carbon and/or energy storage material under conditions of excess carbon source, but with the limitation of nutritional elements, such as nitrogen, phosphorus, sulfur, magnesium or oxygen (Anderson and Dawes,1990; Kranz *et al*., 1997; Poirier *et al*., 1995). PHAs exist as discrete inclusions localized in the cell cytoplasm of the microorganisms, as discovered by Lemoigne in 1926 when granule-like inclusion bodies were observed in *Bacillus megaterium* (Lemoigne, 1926). It has been shown that PHA accumulation can comprise almost 90 % of the bacterial dry cell weight, without causing significant effect to the osmotic pressure in the cell. Accumulation of intracellular granules in polymerized insoluble forms

neither affects the cell function nor cause leakage of the polymer out of the cell (Madison and Huisman, 1999; Verlinden *et al*., 2007).

PHA granules stain specifically with Sudan black or light fluorescent stains such as Nile blue and Nile red (Gorenflo *et al.*, 1999; Kitamura and Doi, 1994; Ostle and Holt, 1982; Spiekermann *et al.*, 1999). PHA granules can be observed as light-refracting granules under phase contrast light microscope, which are common methods adapted for qualitative determination of PHA-producing bacterial strains. Alternatively, ultrastructure observation of thin sections of cells containing PHA granules can be carried out under transmission electron microscope.

In addition to the interest in the roles of PHAs in the environment, there have also been significant efforts to develop PHAs for commercial use. These polymers are gaining world wide attention and are currently being studied extensively due to similarities in terms of material and physical properties, compared with some petroleum-based synthetic plastics. PHAs extracted from bacterial cells show material properties that are similar to polypropylene (Braunegg *et al*., 1998). The chemical and physical properties of PHAs are influenced by the functionalized groups in the side chain of monomers such as, halogen, carboxyl, hydroxyl, epoxyl and phenoxy (Kessler *et al.*, 2001; Kim and Lenz, 2001). The advantages of these materials over petrochemical plastics are that they are natural, renewable and biocompatible, and are degradable via enzymatic reactions by a wide range of microorganisms (Mergaert *et al*., 1993; Steinbüchel, 2001; Sudesh and Iwata, 2008). PHAs are known to be a hundred per cent biodegradable into carbon dioxide and water in aerobic conditions, and into methane in anaerobic conditions (Khanna and Srivastava, 2005). Nonetheless, the high cost of producing bioplastics far above the

price of conventional plastics led to bioplastics being ignored for a long time (Salehizadeh and Van Loosdrecht, 2004).

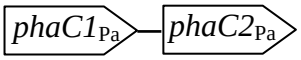
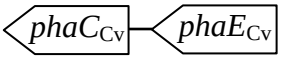
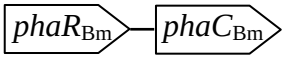
2.2 The key enzyme in PHA biosynthesis: PHA synthase

PHA synthases (PhaC), also referred to as PHA polymerases, are enzymes that catalyze the polymerization of hydroxyacyl-coenzyme A (HA-CoA), provided by precursor pathways, into water insoluble PHA with the concomitant release of CoA (Jendrossek, 2009; Rehm, 2003). Genes encoding these synthases have been identified in numerous species of bacteria (Rehm, 2003; Rehm and Steinbüchel, 2001). PHA synthases are divided into four different classes based on their structure, substrate specificities and subunit composition, as summarized in Table 2.1.

Class I and class II synthases are similar in that they are homodimers with a single subunit (PhaC), but differ their substrate specificities. Class I synthases polymerize only short chain length (SCL, 3 – 5 carbon atoms**)** substrates, while class II synthases prefer medium chain length (MCL, 6 – 14 carbon atoms**)** substrates. Class I and class II synthases are represented by *Cupriavidus necator* and *Pseudomonas aeruginosa*, respectively. Some unique synthases that make SCL-MCL copolymers, such as the PhaC from *Aeromonas caviae* (Fukui and Doi, 1997) and *Chromobacterium* sp. USM2 (Bhubalan *et al*., 2010b), are difficult to classify with this system.

Class III and class IV synthases require two subunits for full activity. In both cases, the PhaC subunit shows homology to the class I and class II synthases. The second subunit (PhaE for class III, PhaR for class IV) is required for full activity, however their roles remain unclear. Class III and class IV synthases are represented by *Chromatium vinosum*, previously known as *Allochromatium vinosum*

Table 2.1: A summary of the different classes of PHA synthases and the representative species for each class.

Class	Gene structure	Subunits	Preferred substrate	Representative species
I		~ 60 – 73 kDa	SCL-HA-CoA	*Cupriavidus necator*
II		~ 60 – 65 kDa	MCL-HA-CoA	*Pseudomonas aeruginosa*
III		PhaC ~ 40 kDa PhaE ~ 40 kDa	SCL-HA-CoA; MCL-HA-CoA	*Chromatium vinosum*
IV		PhaC ~ 40 kDa PhaR ~ 22 kDa	SCL-HA-CoA	*Bacillus megaterium*

(Liebergesell *et al.*, 1991; Yuan *et al.*, 2001), and *Bacillus megaterium* (McCool and Cannon, 1999), respectively.

PHA synthases were discovered to share similar structure and mechanism to bacterial lipases (Jia *et al*., 2000). These enzymes are part of the α/β hydrolase family, and act at the interface between an aqueous solution and a hydrophobic surface. Both lipases and PHA synthases contain a lipase box with the sequence of N-X-X-G-X-C/S-X-G-G which includes the key catalytic residue (serine for lipases, cysteine for PHA synthases). The roles of several catalytic residues in PHA synthases have been determined based on alignments of amino acid sequences of PHA synthases and three dimensional models of synthases. They are the cysteine, histidine, and aspartate residues (C319, H508, and D480) located in the active site of the PHA synthase from *C. necator*. Mutations to any of these residues was found to diminish synthase activity of the wild type synthase (Jia *et al*., 2000; Jia *et al*., 2001).

Experiments have been designed to elucidate the PHA synthase mechanism, many of which utilize synthases from *C. necator* and *C. vinosum* isolated from recombinant *Escherichia coli*. Initial efforts to study PHA synthases were hampered by variable lag phases exhibited by enzymes as well as difficulties in obtaining enzymes purified to homogeneity (Gerngross *et al*., 1994; Haywood *et al*., 1989). It was later discovered that the lag phase could be eliminated by priming PHA synthases with short polyhydroxybutyrate oligomers (Jia *et al*., 2000; Wodzinska *et al*., 1996), and advancements were made in the purification of the PHA synthase from *C. necator*, yielding pure enzymes with up to 90 % homogeneity (Gerngross *et al.*, 1994), followed by successful purification of PHA synthase belonging to *C. vinosum* (Liebergesell *et al.*, 1994). These discoveries and accomplishments allowed

for detailed examination of the substrate specificities of PHA synthases (Yuan *et al*., 2001; Zhang *et al*., 2000).

In the past, substrate specificities of PHA synthases have only been determined in their native environments or in heterologous physiological environments. These are indirect methods, however, and do not provide a good judgement of the substrate specificities due to limitations posed by metabolic pathways that supply monomer units in the particular environment. This is evident based on the differences in the monomer composition of PHA obtained by expression of PHA synthases in various physiological environments. An example is the different substrate range of the PHA synthase from *Chromobacterium* sp. USM2, which was manifested when the PhaC was expressed heterologously in a PHA-negative mutant of *C. necator* (Bhubalan *et al*., 2010b).

Mechanistic studies of PHA synthases have been conducted using various substrates in a bid to understand the substrate specificity of PHA synthases and the formation of PHA copolymers. Doi and co-workers used nuclear magnetic resonance (NMR) to study the distributions of different diad and triad sequences of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] synthesized by *C. necator* (Doi *et al*., 1986). They concluded that the addition of new monomer units to the polymer chain is independent of the unit at the end of the chain and that the reaction proceeds as an ideal random copolymerization. On the other hand, some groups used mutagenesis techniques to alter as well as study the substrate specificity of PHA synthases, notably those belonging to the class II pseudomonads (Matsumoto *et al*., 2006a,b; Takase *et al*., 2003). Mutations affected PHA synthase activity and expression, as well as monomer composition (Nomura and Taguchi, 2007; Taguchi and Doi, 2004). However, the roles of individual residues and the

mechanism of substrate selectivity is still unclear in the absense of the crystal structure of the PHA synthase.

Aside from that, results of studies in which the PHA synthase was incubated with various sulfhydryl inhibitors suggested that the PHA synthase is a sulfhydryl enzyme (Greibel *et al*., 1968). The active-site model of PHA synthase was proposed by Ballard and co-workers based on this, in which two thiol groups were suggested to be involved in locating the hydroxyalkanoate monomers (Ballard *et al*., 1987). Using the purified PHA synthase from *C. necator*, however, it was shown that only one thiol group is essential for catalysis (Gerngross *et al*., 1994). Nevertheless, the most probable reaction mechanism of PHA synthase was postulated to include two thiol groups with the second thiol made available following posttranslational modification via a phophopantethine moiety. Another model proposed that PHA synthases consisting of only one subunit undergo dimerization to form a homodimer, the formation of which was suggested to be responsible for the observed lag phase (Gerngross and Martin, 1995; Gerngross *et al.*, 1994; Liebergesell *et al.*, 1994). On the other hand, PHA synthases consisting of two subunits would form a heterodimer whereby the second thiol is speculated to be provided by a conserved Cys-130 of PhaE subunit from *C. vinosum*.

2.3 *In vivo* substrate provision for PHA synthases

The generation of monomers for PHA synthesis in bacteria is linked with its central metabolism and catabolism of various carbon precursors. Different pathways are involved in the uptake and conversion of various carbon substrates ranging from inexpensive, complex waste effluents, to plant oils, and to alkanes as well as simple carbohydrates into HA-CoA that is subsequently polymerized into PHA (Sudesh *et*

al., 2000). Numerous important pathways such as amino acid metabolism, fatty acid *β*-oxidation, fatty acid *de novo* synthesis and tricarboxylic acid (TCA) cycle have been found to be associated with the production of PHA (Madison and Huisman, 1999; Steinbüchel, 2001; Taguchi *et al.*, 2002a). In most PHA producing bacteria, the synthesis of PHAs other than poly(3-hydroxybutyrate) [P(3HB)] occurs only from precursor substrates structurally related to the hydroxyalkanoate monomers that are to be incorporated into the polymer chains (Anderson and Dawes, 1990; Steinbüchel and Valentin, 1995). Naturally occurring metabolic pathway of PHA biosynthesis varies according to the genus of the bacterium. Three well known metabolic pathways responsible for the synthesis of PHA precursors are the P(3HB) biosynthetic pathway, the fatty acid *β*-oxidation biosynthetic pathway and the *de novo* fatty acid biosynthetic pathway (Aldor and Keasling, 2003; Sudesh and Doi, 2000; Taguchi *et al.*, 2002a).

The model organism widely used for studies on PHA biosynthesis is *C. necator* H16, with the ability to accumulate high levels of P(3HB) when grown in media with plentiful carbon but limited in other essential nutrient. P(3HB) synthesis occurs when CoA thioesters are catalyzed by a PHA synthase, where the polymerization reaction is stereospecific as only (*R*)-3-hydroxyacyl-CoA molecules serve as substrates. P(3HB) biosynthesis pathway (Figure 2.1) is the simplest, which involves three enzymes and their encoding genes. The pathway involves three successive enzymatic reactions, in the order of *β*-ketoacyl-CoA thiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC) (Madison and Huisman, 1999).

In *C. necator*, PHA biosynthesis is initiated through the metabolism of carbohydrates (Anderson and Dawes, 1990). This occurs through the condensation

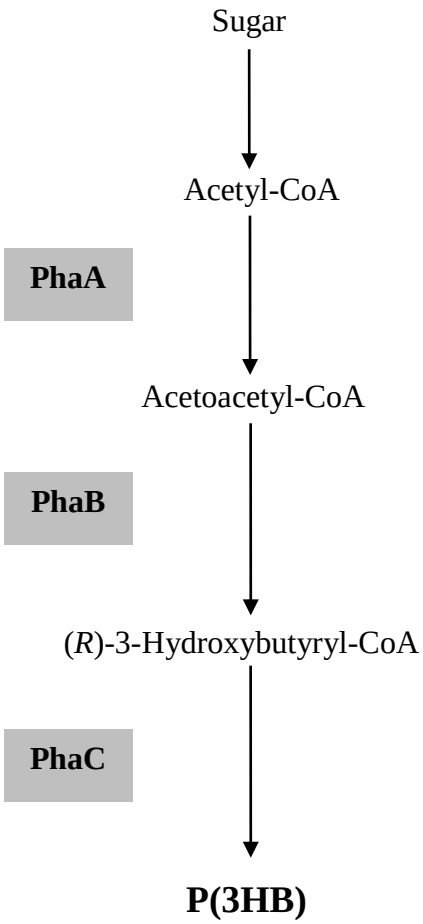


Figure 2.1: P(3HB) biosynthesis pathway (Anderson and Dawes, 1990; Steinbüchel and Lütke-Eversloh, 2003).

of two acetyl-CoA molecules into acetoacetyl-CoA catalyzed by PhaA. Acetoacetyl-CoA is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA (3HB-CoA) by PhaB. In order to complete the process, the synthesized 3HB-CoA has to be polymerized into P(3HB) through a catalytic reaction by PhaC (Anderson and Dawes, 1990). Regulation of the P(3HB) biosynthetic pathway is a complex process which depends on metabolic or environmental conditions involved in the regulation of acetyl-CoA level in the cells (Steinbüchel, 1991; Steinbüchel and Schegel, 1991; Zinn *et al.*, 2001).

Apart from P(3HB), *C. necator* also synthesizes P(3HB-*co*-3HV) (Figure 2.2). Incorporation of the 3-hydroxyvalerate (3HV) monomer into P(3HB) polymer chains is known to improve the properties of P(3HB). P(3HB-*co*-3HV) has lower crystallinity and melting temperature while exhibiting greater flexibility and toughness compared to P(3HB) (Doi *et al*., 1988). P(3HB-*co*-3HV) show isomorphic cocrystallization whereby the 3HB and 3HV units may cocrystallize in a crystalline lattice and the formation of crystal structures depends on the polymeric units containing different compositions of 3HB and 3HV (Bluhm *et al*., 1986). Isomorphic crystallization is a normal phenomenon which can be observed among some natural substances analogous in size and chemical structure such as sodium nitrate, calcium sulfate and barium sulfate.

Supplementation of precursors such as alkanoic acids (Du *et al*., 2001; Khanna and Srivastava, 2007), alkanoates (Le*e et al*., 2008; Shang *et al*., 2004) and alcohols (Park and Damodaran, 1994) with the odd number of carbon atoms leads to the production of the 3HV monomer. If propionic acid is fed, the pathway involved is essentially identical to that for P(3HB) synthesis. In this pathway, propionic acid is initially converted to propionyl-CoA. A distinct 3-ketothiolase in *C. necator*

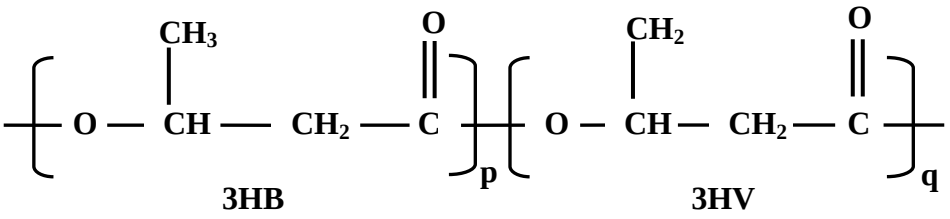


Figure 2.2: Chemical structure of P(3HB-*co*-3HV).

* p and q refers to the number of each repeating unit in the copolymer

(BktB) will then mediate the condensation of acetyl-CoA, generated from the TCA cycle, with propionyl-CoA to form 3-ketovaleryl-CoA. Alternatively, elimination of the carbonyl carbon of propionyl-CoA may occur to form acetyl-CoA, in which case it will condense with another acetyl-CoA to generate an acetoacetyl-CoA. The 3-ketovaleryl-CoA and acetoacetyl-CoA are then reduced to (*R*)-3-hydroxyvaleryl-CoA and (*R*)-3-hydroxybutyryl-CoA, respectively, to be polymerized into P(3HB-*co*-3HV) by PhaC (Braunegg *et al.*, 1998; Doi *et al.*, 1987). The acetyl-CoA which is formed from propionyl-CoA can also be channeled into TCA cycle for cell metabolism.

On the contrary, valeric acid can form valeryl-CoA which is then directly converted to (*S*)-3-hydroxyvaleryl-CoA via *β*-oxidation pathway without being broken down into a shorter chain. Subsequently, (*S*)-3-hydroxyvaleryl-CoA can be converted into 3-ketovaleryl-CoA which is reduced to (*R*)-3-hydroxyvaleryl-CoA and polymerized as described before. A small amount of converted 3-ketovaleryl-CoA may be degraded into one propionyl-CoA and one acetyl-CoA. Production of P(3HB-*co*-3HV) with high 3HV molar fractions using various microorganisms such as *C. necator*, *Chromobacterium violaceum* and *Delftia acidovorans* has been reported (Doi *et al.*, 1988; Loo and Sudesh, 2007; Steinbüchel *et al*., 1993).

Precursors for the synthesis of MCL-PHA monomers, such as 3-hydroxyhexanoate (3HHx), are provided via fatty acid *β*-oxidation. There has been substantial interest in species of the *Aeromonas* genus due to the inherent potential to produce flexible poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] (Figure 2.3) with 3HHx fraction ranging from 3 – 18 mol% (Chen *et al.*, 2001; Doi *et al.*, 1995; Kobayashi *et al.*, 1994; Lee *et al.*, 1999). Fatty acids are initially activated by acyl-CoA synthetase into respective acyl-CoA thioesters before

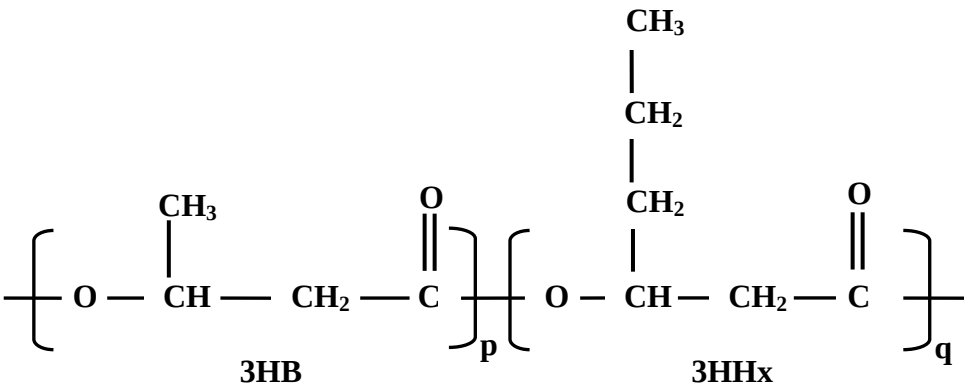


Figure 2.3: Chemical structure of P(3HB-*co*-3HHx).

* p and q refers to the number of each repeating unit in the copolymer

they enter the *β*-oxidation pathway. In the *β*-oxidation pathway, acyl-CoA is oxidized into enoyl-CoA by acyl-CoA dehydrogenase which is then converted into (*S*)-3-hydroxyacyl-CoA by enoyl-CoA hydratase. Oxidation of (*S*)-3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydrogenase results in the formation of 3-ketoacyl-CoA, which is cleaved by *β*-ketothiolase to form acetyl-CoA and acyl-CoA. However, this newly generated acyl-CoA is shorter by two carbon atoms as compared to the acyl-CoA that was present during the first cycle. In the case of fatty acids with even carbon number, further cycles go on until the original acyl-CoA is fully converted into acetyl-CoA (Potter and Steinbüchel, 2006; Steinbüchel and Lütke-Eversloh, 2003).

The *β*-oxidation pathway intermediates including enoyl-CoA, (*S*)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA, can serve as precursors for MCL-PHA synthesis. However, none of these intermediates are present in the form accepted as substrate by the PHA synthase. Therefore, an additional step is required to convert these intermediates into (*R*)-3-hydroxyacyl-CoA, which can be polymerized by PHA synthase into corresponding monomers (Steinbüchel and Lütke-Eversloh, 2003; Suriyamongkol *et al.*, 2007). Three different enzymes are responsible for the conversion; epimerase, (*R*)-specific enoyl-CoA hydratase and 3-ketoacyl-CoA reductase. Epimerase catalyzes the conversion of 3-hydroxyacyl-CoA of the (*S*)-isomer into (*R*)-isomer. On the other hand, (*R*)-specific enoyl-CoA hydratase functions in converting enoyl-CoA into (*R*)-3-hydroxyacyl-CoA. The 3-ketoacyl-CoA reductase reduces 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA. Meanwhile, acetyl-CoA could be channeled either to the TCA cycle, for fatty acid synthesis or formation of P(3HB). A second route for MCL-PHA synthesis in microorganisms is through the use of intermediates of the *de novo* fatty acid biosynthesis pathway

(Steinbüchel and Lütke-Eversloh, 2003; Tsuge, 2002). Fatty acid synthesis and *β*-oxidation display similar chemistries but are regulated by different enzymes (Figure 2.4).

Polymers with the physical and mechanical properties of both P(3HB-*co*-3HV) and P(3HB-*co*-3HHx) copolymers, namely poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HV-*co*-3HHx)] (Figure 2.5), have also been produced. Bacteria strains investigated for the production of this terpolymer include *Rhodospirillum rubrum* (Brandl *et al.*, 1989), *Rhodocyclus gelatinosus* (Liebergesell *et al.*, 1991) and *Rhodococcus* sp. (Anderson *et al.*, 1990). P(3HB-*co*-3HV-*co*-3HHx) can be produced from even carbon numbered fatty acids as the main carbon source and valeric acid or propionic acid as 3HV precursors (Figure 2.6).

2.4 Diversity in monomer constituents and properties of PHA

PHAs are linear polyesters made up of 3-hydroxyalkanoates with an alkyl group positioned at C3 (Figure 2.7). The type of PHA is dependent on the R and x number in the chemical structure, as shown in Table 2.2. PHA that occurs naturally in microorganisms is P(3HB).

PHA can be categorized into three classes, as such: (i) SCL-PHA consisting of monomers with carbon number in the range of 3 to 5, (ii) MCL-PHA consisting of monomers with carbon number in the range of 6 to 14, and (iii) SCL-MCL PHA containing monomers with carbon number in the range of 3 to 14.

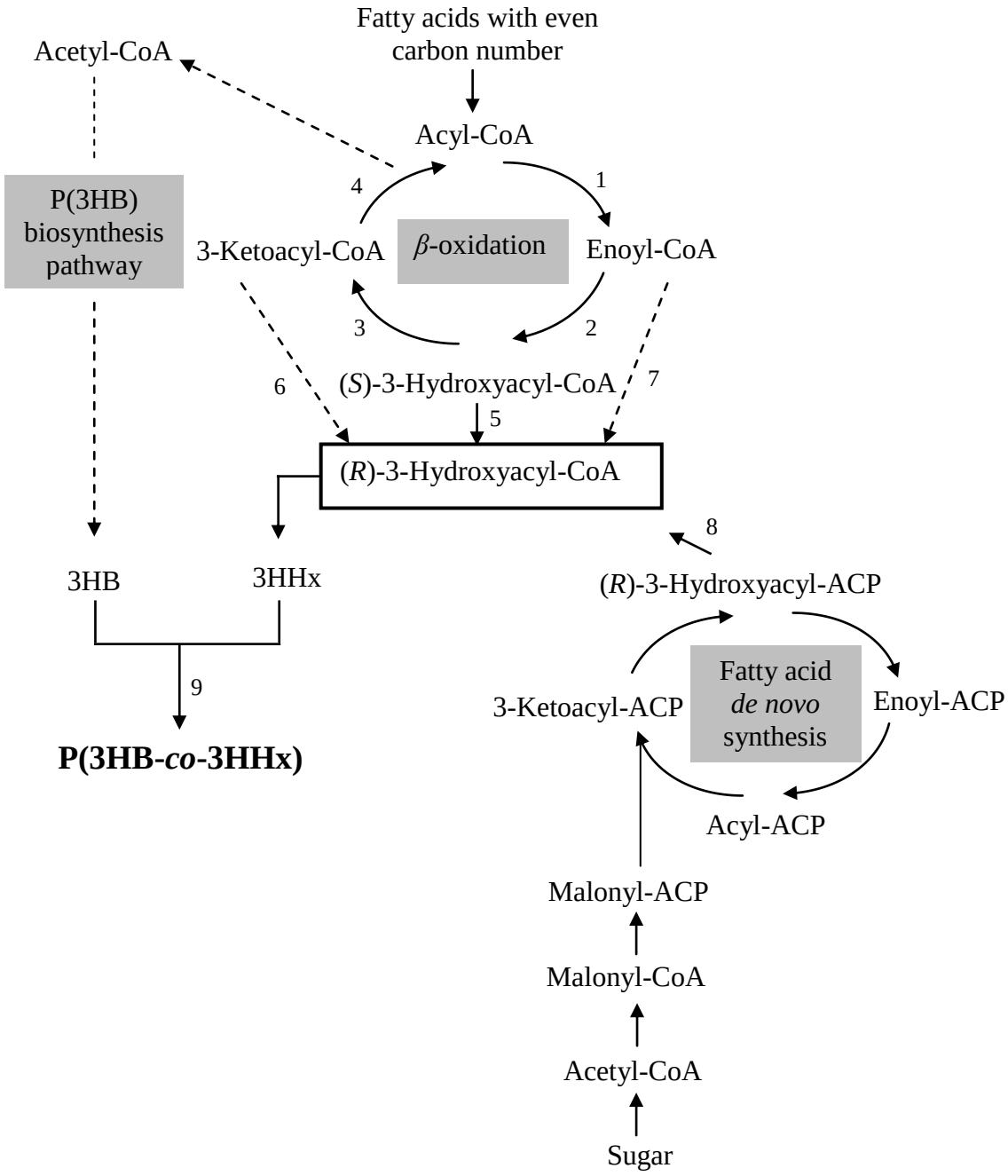


Figure 2.4: *β*-oxidation and *de novo* fatty acid pathways for the biosynthesis of MCL-PHA **(**Sudesh *et al.*, 2000; Suriyamongkol *et al.*, 2007) and subsequent polymerization into P(3HB-*co*-3HHx). Enzymes: 1. acyl-CoA dehydrogenase; 2. enoyl-CoA hydratase; 3. 3-hydroxyacyl-CoA dehydrogenase; 4. 3-ketoacyl-CoA thiolase; 5. epimerase; 6. 3-ketoacyl-CoA reductase; 7. (*R*)-specific enoyl-CoA hydratase; 8. (*R*)-3-hydroxyacyl-ACP-CoA transferase; 9. PHA synthase.

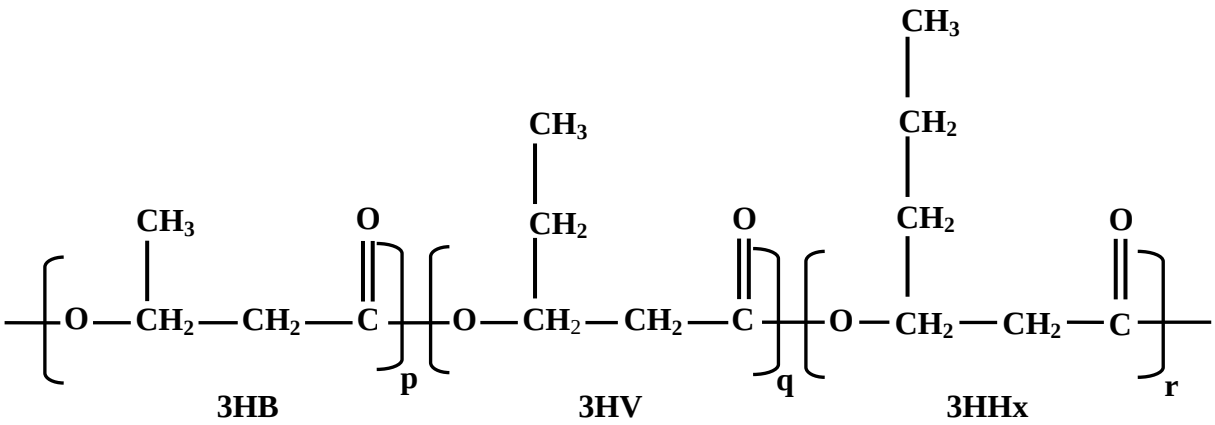


Figure 2.5: Chemical structure of P(3HB-*co*-3HV-*co*-3HHx).

* p, q and r refer to the number of each repeating unit in the terpolymer

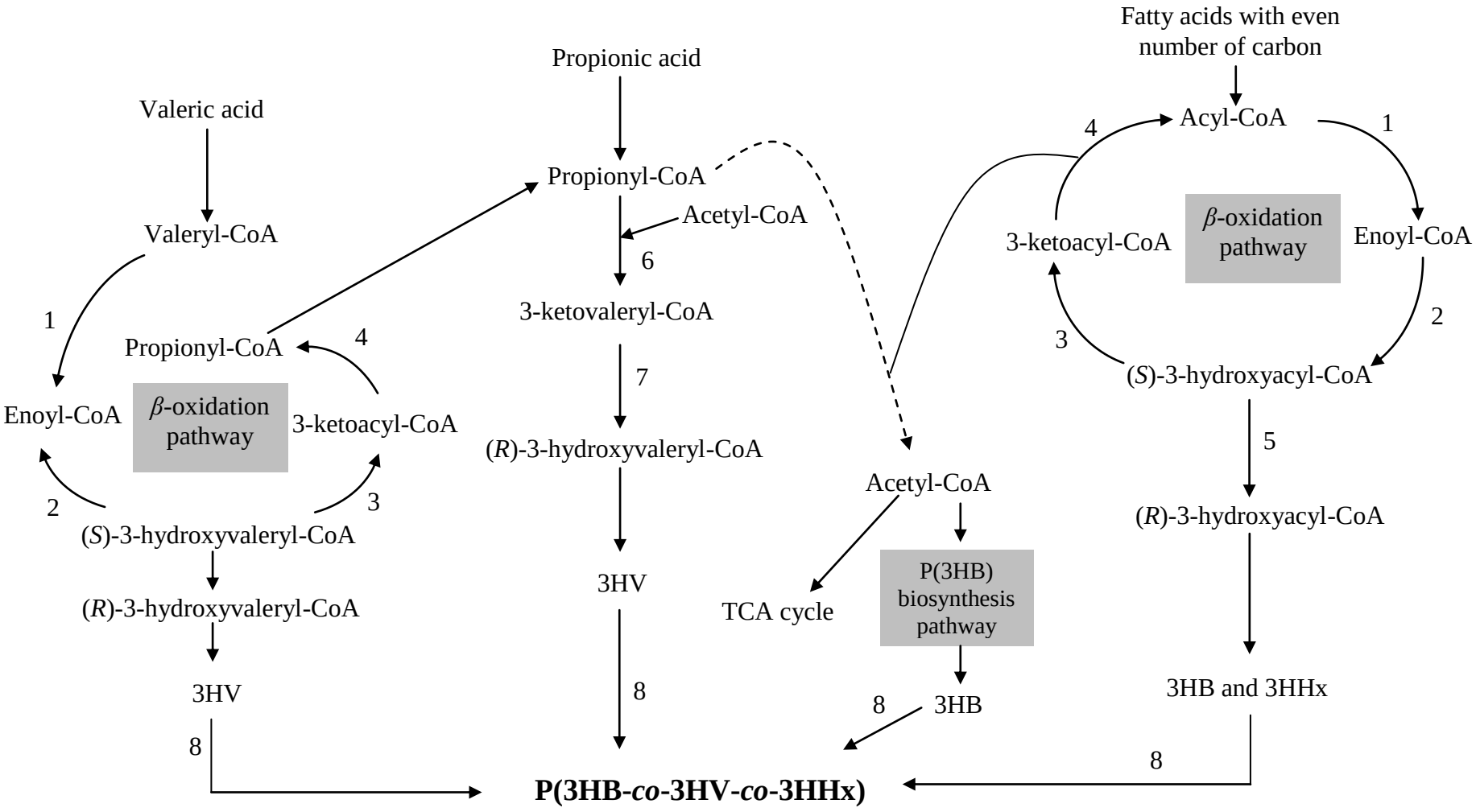


Figure 2.6: Proposed pathway for the biosynthesis of P(3HB-*co*-3HV-*co*-3HHx) from fatty acids with even number of carbon as the main carbon source and valeric acid or propionic acid as 3HV precursors (Bhubalan *et al.*, 2008). Enzymes: 1. acyl-CoA dehydrogenase; 2. enoyl-CoA hydratase; 3. 3-hydroxyacyl-CoA dehydrogenase; 4. 3-ketoacyl-CoA thiolase; 5. epimerase; 6. *β*-ketothiolase; 7. NADPH-dependent acetoacetyl-CoA reductase; 8. PHA synthase.

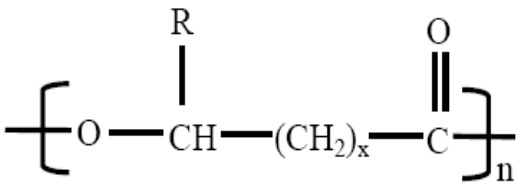


Figure 2.7: Chemical structure of PHA

*n indicates the number of repeating units

Table 2.2: Various types of hydroxyalkanoate monomer formed with different R and x values.

X	R side chain	Type of monomer
1	Methyl	3-hydroxybutyrate; 3HB
	ethyl	3-hydroxyvalerate; 3HV
	propyl	3-hydroxyhexanoate; 3HHx
2	Hydrogen	4-hydroxybutyrate; 4HB
3	Hydrogen	5-hydroxyvalerate; 5HV

* R and x determine the type of hydroxyalkanoate monomer unit formed