CHARACTERIZATION OF THE DEPOLYMERIZING ACTIVITY OF COMMERCIAL LIPASES AND ANIMAL ORGAN EXTRACTS USING A SIMPLE POLYHYDROXYALKANOATE-BASED MICROASSAY

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UNIVERSITI SAINS MALAYSIA

2015

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by

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Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

September 2015

ACKNOWLEDGEMENTS

First of all, I would like to express my greatest appreciation to my supervisor, Prof. Dr. K. Sudesh Kumar for the continuous support throughout my master study. His guidance and patience had helped me a lot in my research project.

Besides that, I would like to thank the seniors and lab members of Ecobiomaterial Research Lab who had provided idea and suggestions on my project especially Dr. Diana Ch'ng Hooi Ean. She had assisted me on biosynthesis of P(3HB-*co*-4HB) and lipase activity assays that were important in my research. I would also like to express my gratitude to Ms. Ong Soo Peng who had helped me on biosynthesis of the polymer.

I am also thankful to MyBrain 15 for solving my financial problems so that I could concentrate on my study. I would like to show my gratitude to Prototype Research Grant Scheme (PRGS) and Dana Inovasi Awal Universiti Sains Malaysia for my research funding.

Last but not least, I would like to show my deepest thankful to my family members who had given me spiritual support and encouragement throughout my research study.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL NAME
PHA	Polyhydroxyalkanoate
4HB	4-hydroxybutyrate
3HP	3-hydroxypropionate
5HV	5-hydroxyvalerate
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PHB	Poly(3-hydroxybutyrate)
PhaA	3-ketoacyl-CoA thiolase
PhaB	NADH/NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
C_4	Molecule containing 4 carbon atoms
C_6	Molecule containing 6 carbon atoms
C_8	Molecule containing 8 carbon atoms
C_{12}	Molecule containing 12 carbon atoms
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
Scl	Short chain length
Mcl	Medium chain length
Lcl	Long chain length
4HBA	4-hydroxybutyric acid
ɣ-BL	ɣ-butyrolactone
1,4-BD	1,4-butanediol

TLC	Thin layer chromatography
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ELISA	Enzyme-linked immuno assay
FT-IR	Fourier transform infrared spectroscopy
NA	Nutrient agar
NB	Nutrient broth
NM	Nitrogen-free mineral
OD	Optical density
CME	Caprylic methyl ester
^{1}H NMR	Proton nuclear magnetic resonance
K	GC constant
A_{3HB}	Area of GC peak for 3HB
A_{gamma}	Area of GC peak for ɣ-butyrolactone
A_{4HB}	Area of GC peak for 4HB
k	Constant for ɣ-butyrolactone peak
m	Constant for 4HB peak
A_{CME}	Area of GC peak for CME
PBS	Phosphate saline buffer
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
SEM	Surface electron microscope
SDS	Sodium dodecyl sulfate

*p*NPL	*p*-nitrophenyl laurate
PVA	Polyvinyl alcohol
DMSO	Dimethyl sulfoxide
GLM	General linear model
CMC	Critical micelle concentration

LIST OF UNITS AND SYMBOLS

UNITS AND SYMBOLS	FULL NAME
°C	Degree Celcius
ɣ	Gamma
%	Percentage
mol%	Mole percent
g/cm^3	Gram per cube centimeter
MPa	Megapascal
α	Alpha
β	Beta
kDa	Kilo Dalton
nm	Nanometer
µmol	Micromole
µg/assay	Microgram per assay
cm^{-1}	Per centimeter
M	Molar
psi	Pounds per square inch
cm	Centimeter
g/L	Gram per liter
mL	Milliliter
rpm	Revolutions per minute
v/v	Volume per volume
w/v	Weight per volume
g	Gravity
mM	Millimolar

mg	Milligram
kPa	Kilopascal
mL/min	Milliliter per minute
g	Gram
mm	Millimeter
mg/mL	Milligram per milliliter
U/mg	Unit per milligram
µg/ mL	Microgram per milliliter
Cu^{2+}	Copper ion
Ni^{2+}	Nickel ion
Ca^{2+}	Calcium ion
Mg^{2+}	Magnesium ion
Mn^{2+}	Manganese ion
K_2HPO_4	di-Potassium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate
$FeSO_4 \cdot 7H_2O$	Iron (II) sulfate
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride
$CoSO_4 \cdot 7H_2O$	Cobalt (II) sulfate
$CaCl_2 \cdot 2H_2O$	Calcium chloride
$CuCl_2 \cdot 2H_2O$	Copper (II) chloride
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate
Gly	Glycine
Ser	Serine
KCl	Potassium chloride

Na_2HPO_4	di-Sodium hydrogen phosphate
$NaHCO_3$	Sodium hydrogen carbonate
Na_2SO_4	Sodium sulfate
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
NaCl	Sodium chloride
kV	Kilovolt
p	Para
µmol min^{-1} mL^{-1}	Micromole per minute per milliliter
µmol	Micromole
min	minute

PENCIRIAN AKTIVITI DEPOLIMERASI OLEH LIPASE KOMERSIAL DAN EKSTRAK ORGAN-ORGAN HAIWAN DENGAN PENGGUNAAN MIKROASAI RINGKAS YANG BERASASKAN POLIHIDROKSIALKANOAT

ABSTRAK

Lipase ialah enzim yang digunakan dalam pelbagai aplikasi dan aktivitinya perlu diuji sebelum aplikasi. Namun begitu, kebanyakkan asai aktiviti lipase memerlukan bahan kimia toksik dan persediaan yang rumit. Oleh itu, poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] digunakan sebagai substrat pepejal kerana ia dapat diuraikan oleh lipase. Substrat ini boleh diuraikan secara semula jadi dan tidak memerlukan bahan kimia yang bertoksik. Dalam kajian ini, 2.0 ± 0 g/L sel mengandungi 28 ± 2 % of PHA dengan 92 ± 1 mol % of 4HB dalam PHA telah dibiosintesis. Kestabilan filem P(3HB-*ko*-4HB) dinilai dengan menyimpan filem tersebut di bawah keadaan yang berbeza iaitu di ketuhar vakum, balang pengering dan meja makmal selama 15 bulan. Filem tersebut paling stabil dalam balang pengering yang mengandungi gel silika. Aktiviti depolimerasi lipase komersial dan ekstrak organ haiwan telah diuji secara mikroasai dengan menggunakan P(3HB-*ko*-4HB) sebagai substrat. Penghasilan aktiviti depolimerasi lipase yang lebih tinggi adalah dari pH 6 hingga pH 8 dan suhu lebih daripada 30 °C tapi kurang daripada 50 °C . Ion logam dan detergen dengan pelbagai kepekatan bagi lipase komersial menunjukkan aktiviti depolimerasi yang berbeza. Contohnya, aktiviti depolimerasi dirangsang oleh 1 mM ion kalsium dalam lipase dari pankreas khinzir dan *Rhizopus arrhizus* tetapi ion tersebut menghalang aktiviti lipase lain. Untuk lipase dari *Candida rugosa*, aktiviti depolimerasi direncat oleh 0.1 % Triton X-100 tetapi dirangsang oleh 0.1 % SDS, Tween 20 dan Tween 80. Selain lipase komersial,

aktiviti depolimerasi juga diuji dengan menggunakan ekstrak organ tikus dan ayam. Duodenum dan pankreas dari kedua-dua tikus dan ayam menunjukkan aktiviti depolimerasi atas filem P(3HB-*ko*-4HB). Buat kali pertama, ujian ini telah membuktikan penglibatan enzim dalam degradasi PHA ini dalam haiwan. Lipase dari pankreas adalah enzim yang paling mungkin terlibat dalam degradasi PHA.

CHARACTERIZATION OF THE DEPOLYMERIZING ACTIVITY OF COMMERCIAL LIPASES AND ANIMAL ORGAN EXTRACTS USING A SIMPLE POLYHYDROXYALKANOATE-BASED MICROASSAY

ABSTRACT

Lipase is an enzyme that is widely used in different applications and it is important to screen its activity before application. However, most of the available lipase activity assays require toxic chemicals and tedious preparation. Therefore, a solid substrate known as poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] is used and it can be degraded by lipases. This substrate is biodegradable and does not require toxic chemical. In this study, 2.0 ± 0 g/L of cells consisting 28 ± 2 % of PHA with 92 ± 1 mol % of 4HB were produced through biosynthesis. The stability of P(3HB-*co*-4HB) films was evaluated by storing them in different locations, in vacuum oven, desiccators and bench for 15 months. The film was found to be the most stable in desiccators containing silica gel. The depolymerizing activity of different known commercial lipases and animal organ extracts using P(3HB-*co*-92 mol% 4HB) as substrate was investigated via microassay. The depolymerizing activity of lipases on P(3HB-*co*-4HB) was higher in range of pH 6 to 8 and temperature above 30 °C but lower than 50 °C. Addition of metal ions and detergents in different concentrations had variable effects on the depolymerizing activity of commercial lipases. For instance, 1 mM of calcium ions stimulated depolymerizing activity of lipase from porcine pancreas and *Rhizopus arrhizus* but inhibited the activity of other lipases. For lipase from *Candida rugosa*, 0.1 % of Triton X-100 inhibited its depolymerizing activity while 0.1 % of SDS, Tween 20 and Tween 80 stimulated its activity. Besides commercial lipases, the crude extracts of different organs from mice and chicken were also screened for the presence of depolymerizing

activity. The duodenum and pancreas from both mice and chicken showed depolymerizing activity on the P(3HB-*co*-4HB) film. This for the first time has produced a direct evidence for the involvement of enzymes in the degradation of this PHA in animals. Lipase is the most likely enzyme from pancreas that was involved in the degradation.

CHAPTER 1

INTRODUCTION

1.0 Introduction

Lipase is a known enzyme that is widely used in industries. Generally, enzymes are biological catalysts which increase the rates of reactions under certain conditions such as temperature, pH, cellular concentration and stability in organic solvent (Ghosh *et al.,* 1996). Each enzyme has its own optimal temperature and pH range that affects its structural integrity. The three-dimensional structures of the enzymes contribute to the effectiveness of the enzyme reaction and catalysis. Enzymes also participate in chemical modification. An important characteristic of enzymes is that they are highly selective and absolute specific to certain molecules, known as substrates (Dalziel, 1957).

Lipases are known as glycerol ester hydrolases which act on the carboxyl ester bonds in triacylglycerol's to produce glycerol and free fatty acids. They catalyze the hydrolysis and synthesis of different substrates (Li & Zhang, 2005). In fact, lipases play vital role in biotechnology and biomedical fields. Lipases, especially microbial lipases, are widely used in industrial applications such as food and flavor industry, detergents, bioremediation, fine chemical, baking, leather and paper industry (Hasan *et al.,* 2006; Jaeger & Eggert, 2002). Typically, lipases can be obtained from microorganisms (Olempska-Beer *et al.,* 2006), plants (Bhardwaj *et al.,* 2001) and animals (Carriere *et al.,* 1994).

It is worthwhile to determine the lipase activity to study its effectiveness. Lipase activity can be measured by various methods such as titrimetry and

colorimetry but these methods are not eco-friendly and require the use of toxic chemicals, specific skills as well as expensive instruments (Stoytcheva *et al.*, 2012).

Polyhydroxyalkanoates (PHAs) are microbial polyesters synthesized by various types of bacteria such as *Cupriavidus necator*, *Alcaligenes* spp., *Pseudomonas* spp., *Bacillus* spp., *Aeromonas hydrophila* and *Burkholderia sacchari* (Verlinden *et al.*, 2007). Much research works are ongoing to develop PHAs as biodegradable plastics. Besides the potential use of PHAs as an ecofriendly plastic material, other types of niche applications for PHAs have also been proposed. For example, Sudesh *et al*. have proposed the use of PHA as cosmetic facial oil blotting film (Sudesh *et al.*, 2007). Besides that, PHA is also used as probe for the detection of microbial activities since it can be degraded by microbes via enzymatic reaction (Sudesh, 2010). PHAs containing 4-hydroxybutyrate (4HB) monomer are especially attractive as bioabsorbable material (Martin & Williams, 2003). PHAs containing 4HB, 3-hydroxypropionate (3HP) or 5-hydroxyvalerate (5HV) share a unique property, whereby they are hydrolysable by the enzyme lipase (Mukai *et al.*, 1993). The P(3HB-*co*-4HB) is commonly used in medical field as scaffold and suture owing to its bioabsorbable properties (Ying *et al.*, 2008). Recently, this copolymer has also been used as substrate for lipase activity qualification and quantification due to its ability to be degraded by lipase (Ch'ng & Sudesh, 2013).

P(3HB-*co*-4HB) is a solid substrate that is applied in screening of lipase activity. It can be classified as a ‘go green’ method due to its biodegradability. This method of lipase activity screening uses the P(3HB-*co*-4HB) copolymer as substrate (Ch'ng & Sudesh, 2013). It is simple and does not require expensive instruments such as spectrophotometer. Besides that, the copolymer can be discarded after screening without causing any environmental issues due to its biodegradability.

However, further studies are required to make this lipase degrading substrate more reliable as lipase activity assay kit to suit its applications in industries, medical and academic fields. Herein, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was produced for screening of lipase depolymerizing activity. Different conditions such as pH, temperature and addition of metal and detergent on lipase depolymerizing ability were investigated. In addition, depolymerizing activities from animal organ extracts were also screened using P(3HB-*co*-4HB) in order to detect the presence of enzymes with this ability. On top of that, studies were also carried out to determine a proper storage location in order for the polymer to be stored over a long period of time.

1.1 Research objectives

The objectives of this study are as follows:-

a) To evaluate the stability of P(3HB-*co*-4HB) film for lipase depolymerizing activity at different storage locations.
b) To investigate the depolymerizing activity of commercial lipases on P(3HB-*co*-4HB) film under different conditions.
c) To evaluate the ability of the P(3HB-*co*-4HB) film for detection and quantification of depolymerizing activities from animal organ extracts.

CHAPTER 2

LITERATURE REVIEW

2.0 Literature Review

2.1 Lipase

Lipase is a water-soluble and ubiquitous enzyme which is found in various organisms including bacteria, fungi, plants and animals. Lipase is also known as triacylglycerol acylhydrolase (E.C. 3.1.1.3), a unique class of hydrolase. Lipase is able to catalyze the hydrolysis or synthesis of different substrates that are not soluble in water. Some examples of the substrates include phospholipids, triglycerides and cholesteryl esters (Wong & Schotz, 2002). Lipase acts on the ester bonds in the long-chain triacylglycerides to form monoacylglyceride, diacylglyceride, glycerol and free fatty acids under aqueous conditions (Thomson *et al.*, 1999). The reaction for lipases is reversible and can also lead to acidolysis, alcoholysis, aminolysis, esterification, and interesterification (Gupta *et al.*, 2004).

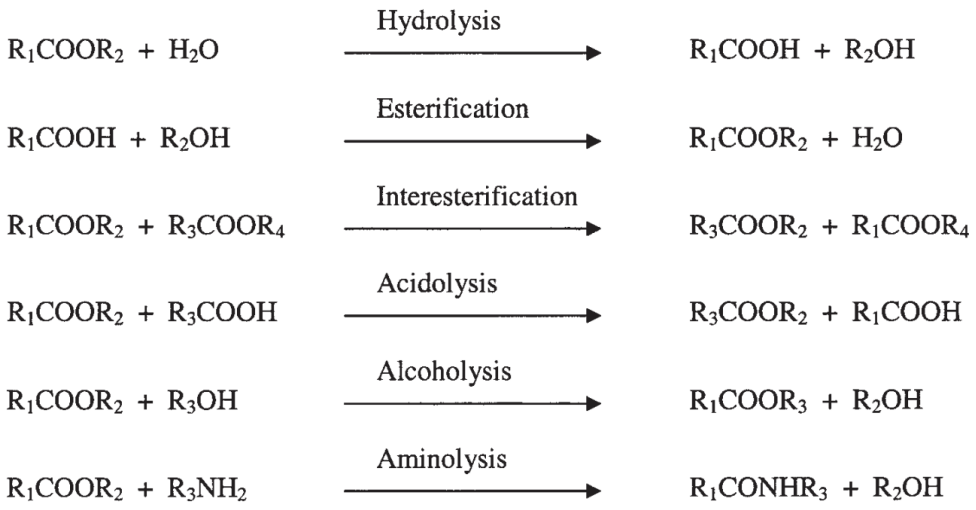


Figure 2.1 Different reactions catalyzed by lipase. R_1, R_2, R_3 and R_4 represent different side chains that attached to functional group of the molecule (Houde *et al.*, 2004).

2.1.1 Sources and Classification of Lipase

Lipases are ubiquitous enzymes that are abundant in nature. Lipases are produced by microorganisms, plants and animals especially in bacteria, yeasts and fungi (Haki & Rakshit, 2003). Although lipases are classified into different groups based on their amino acid sequences and biological properties, they possess similar architecture which is the α/β hydrolase fold. The α/β hydrolase fold enzymes are simple hydrolytic enzymes and have conserved α/β structural core. These enzymes consist of parallel and eight-stranded β sheet surrounded by α helices. There is catalytic triad to conserve the structural features of the fold. Catalytic residues are made of serine which is known as nucleophile, acidic residue and conserved histidine residue. These catalytic residues constitute highly conserved catalytic triad. Consensus sequence of Gly-Xaa-Ser-Xaa-Gly is shared by lipases and the 'X' can

constituted of any amino acid residue (Ollis *et al.*, 1992). In comparison with lipases produced by plants and animals, microbial lipases or lipases from thermophiles are more thermally stable and could resist chemical denaturation. Thus, microbial lipases are more preferred by industries and are widely used in the fields of biotechnology and organic chemistry. However, lipases produced from plants and animals are also essential.

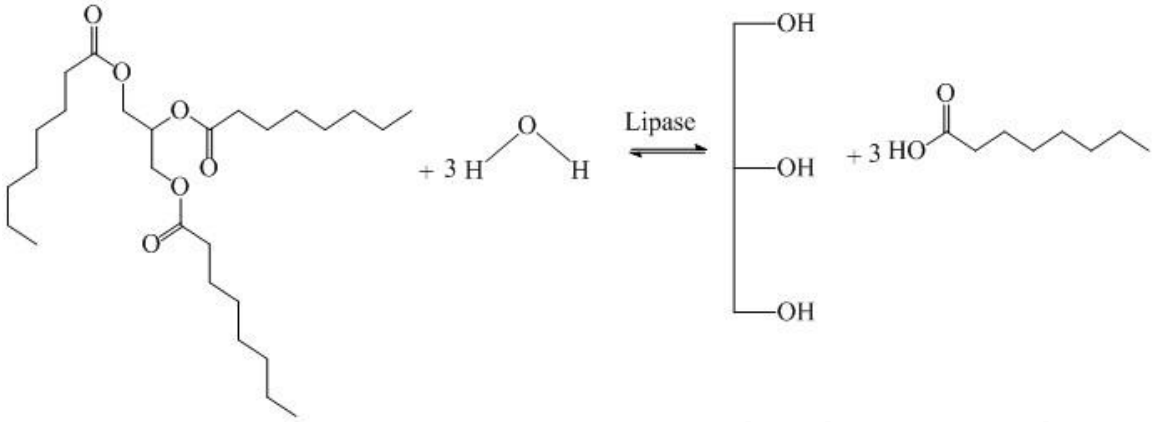


Figure 2.2 Hydrolytic and synthetic reactions of lipase. Lipase acts on the ester bonds in triacylglycerol in the presence of water to produce glycerol and fatty acid.

2.1.2 Properties of lipase

As described in Section 2.1, lipase is water soluble while its substrate is water insoluble. Lipases catalyze hydrolysis of ester bonds at the interfaces between insoluble substrate and soluble lipase. Lipase activity assay is difficult to be measured at this interface. Thus, emulsion of substrate is normally used. However, the factors of emulsion that affect the interface are clarified because some

intermediates formed during lipase reaction accumulate at the interface which prevent lipase to access further substrate (Jensen, 1983).

Lipases from different sources were purified and studied (Macrae, 1983). The purified lipases are commonly acidic glycoproteins and have molecular weights ranging from 20 to 60 kDa. Although the lipases are purified, they still contain carbohydrate side chains. These side chains are not associated with lipases' catalytic activity and do not affect the lipase activity (Semeriva *et al.,* 1969).

Generally, lipases are stable at room temperature in neutral aqueous solutions. Most of them lose activity above 40 ℃ but some are resistant towards heat. For instances, lipases from *Aspergillus niger* and *Chromobacterium viscosum* are stable even at 50 ℃ (Fukumoto *et al.*, 1963; Yamaguchi *et al.*, 1973). Besides that, lipases have broad range of pH with maximum activity from pH 6 to 8. They also show high activity from pH 5 to 9. Metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} sometimes act as either stimulators or inhibitors. Ca^{2+} generally stimulates lipase reaction by removing inhibitory factors such as fatty-acid anions (Sharma *et al.*, 2001).

Esterase and lipase are somewhat similar but in terms of functionality, esterases hydrolyze soluble esters that consist of less than 10 carbon atoms while lipases hydrolyze long chain fatty acid esters which contain more than 10 carbon atoms (Macrae, 1983). There are generally two types of lipases based on its action on substrate molecules. They are nonspecific lipase and regiospecific lipase. Nonspecific lipase acts on all positions of the triacylglycerol molecule while regiospecific lipase acts on the outer positions of the triacylglycerol molecule. Regiospecific lipases are preferred over the nonspecific lipases because the former can produce unique and functional lipids (Sonnet & Gazzillo, 1991).

2.1.3 Applications of lipase

There are various applications of lipase in industrial and medical fields. In fat and oil processing, lower value lipid can be altered by lipase to form higher value fat. For example, phospholipids in vegetable oil are removed using phospholipases (Clausen, 2001). Black tea is also processed using lipase. Lipase is used to breakdown lipids and initiates production of volatile compounds with flavoring (Latha & Ramarethinam, 1999). Oleochemicals are processed by reactions that require high thermal energy and expensive equipments. These processes can be conserved as lipase plays a role in process initiation and ensure high productivity (Ghosh *et al.*, 1996; Saxena *et al.*, 1999). Lipases are also used to improve the food quality by producing flavors and modifying its structure *via* interesterification and transesterification (Reetz, 2002). Besides food industry, lipase is widely used in detergent. Detergent containing lipase is able to remove oily stain on fabrics (Jeon *et al.*, 2009). For paper and pulp industry, hydrophobic components that cause difficulties in paper and pulp making can be removed by lipase (Jaeger & Reetz, 1998). Lipases are also used in bioremediation. Wastes of lipid produced by factories and restaurants can be degraded by lipases to reduce environmental pollution (Pandey *et al.*, 1999). In medical fields, lipase is used as probe and diagnostic tool to detect certain diseases such as pancreatic injury. The level of lipase in blood serum can be investigated using lipase activity assays (Lott & Lu, 1991; Munoz & Katerndahl, 2000).

Table 2.1 Commercially available lipases and their industrial applications (Houde *et al.*, 2004)

Industry	Application	Trade name[a]	Supplier
Dairy		Lipase A "Amano" 6 (*Aspergillus niger*)	Amano
		Lipase M "Amano" 10 (*Mucor javanicus*)	Amano
		Lipase F-AP15 (*Rhizopus oryzae*)	Amano
		Lipase AY "Amano" 30 (*Candida rugosa*)	Amano
		Lipase G "Amano" 50 (*Penicillium camembertii*)	Amano
		Piccnate (*Mucor miehei*)	Gist-Brocades
	EMC production (cheddar-type flavors)	Lipomod™ 187P-L187P (fungal lipases)	Biocatalysts
	EMC production (cheddar-type flavors)	Lipomod™ 224P-L224P (porcine pancreas)	Biocatalysts
	EMC production (blue-type flavors)	Lipomod™ 338P-L338P (*Penicillium roquefortii*)	Biocatalysts
	EMC production	Lipomod™ 34P-L034P (*Candida cylindracea* [*rugosa*])	Biocatalysts
	Cheese flavor (cheddar-type flavors)	Lipomod™ 621P-L621 (*Penicillium* sp./*Aspergillus* sp.)	Biocatalysts
	EMC production (cheddar-type flavors)	Lipomod™ 29P-L029P (*Candida cylindracea* + porcine pancreas)	Biocatalysts
	Cheese-flavor enhancement	**Palatase**® (*Rhizomucor miehei*)	Novozymes
Oil and fat		Lipase A "Amano" 6 (*Aspergillus niger*)	Amano
		Lipase M "Amano" 10 (*Mucor javanicus*)	Amano
		Lipase G "Amano" 50 (*Penicillium camembertii*)	Amano
		Lipase F-Ap15 (*Rhizopus oryzae*)	Amano
		Lipase AY "Amano" 30 (*Candida rugosa*)	Amano
		Newlase F (*Rhizopus niveus*)	Amano
	Interesterification of vegetable oil	**Lipozyme® TL IM**	Novozymes
Pharmaceutical	Pharmaceutical ingredient	Lipase MY (*Candida cylindracea* [*rugosa*])	Meito Sangyo
	Synthesis of chiral compounds	Lipase ALC, Lipase ALG (*Achromobacter* sp.)	Meito Sangyo
	Synthesis of chiral	Lipase PLC, Lipase PLG,	Meito

	compounds	Lipase QLC, Lipase QLG (*Alcaligenes* sp.)	Sangyo
		Lipase SL (*Burkholderia cepacia*)	Meito Sangyo
		Lipase TL (*Pseudomonas stutzeri*)	Meito Sangyo
		Lipase UL (*Rhizopus* sp.)	Meito Sangyo
	Chiral synthesis	Lipase AK "Amano" (*Pseudomonas fluorescens*)	Amano
	Chiral synthesis	Lipase AYS "Amano" (*Candida rugosa*)	Amano
		Lipase PS "Amano" (*Pseudomonas cepacia*)	Amano
Detergent		Lipolase®, Lipolase® Ultra, Lipo Prime™, Lipex® (*Thermomyces lanuginosus*)	Novozymes
Baking	Improvement of dough texture and color	Lipomod™ 627P-L627P (*Rhizopus oryzae*)	Biocatalysts
	Emulsifier	**Lipopan® F**	Novozymes
Leather	Liming	**NovoLime®** (with protease)	Novozymes
	Fat dispersion	**Greasex®, NovoCor® AD**	Novozymes
Cosmetics	Production of isopropyl myristate (cosmetic component)	**Novozym® 435** (*Candida antarctica* B)	Novozymes
Paper	Control of pitch	**Resinase®** (*Candida rugosa*)	Novozymes
Noodles/pasta	Improvement of quality of noodles and wheat-based pasta products	**Noopazyme®**	Novozymes
Miscellaneous	Dietary supplement	Lipase L036P-L036P (*Rhizopus oryzae*)	Biocatalysts
	Dietary supplement	Lipase F-DS (*Rhizopus oryzae*)	Amano
	Delipidation of egg white	Lipomod™ 34P-L034P (*Candida cylindracea*)	Biocatalysts
	Various uses	Lypolyve AN (*Aspergillus niger*)	
	Various uses	Lypolyve CC (*Candida cylindracea*)	

[a] represents 'lipases in bold are recombinant'

2.2 Lipase activity assays

There are numerous methods available on measuring lipase activity. It is crucial to develop more sensitive and fast methods for lipase activity assay (Stoytcheva *et al.*,

2012). Typically, lipase activity can be determined by the release of glycerol or fatty acid from fatty acid esters and the changes occur in oil and water interface.

2.2.1 Plate assay

Also known as gel-diffusion assay, plate assay uses tributyrin agar plate for lipase screening. Tributyrin is hydrolyzed by either esterase or lipase to form zone. Olive oil is supplemented into agar plates to screen colonies that are able to produce lipase (Hube *et al.*, 2000). Indicators such as Nile Blue Sulfate and Victoria Blue are also added to form colored zone. The colored zone is formed when the indicator and acids turn into complexes. This method is considered as fast screening of lipase production by growing microorganisms that are able to produce lipase on the agar plate. However, acids by-products may be produced during the growth of microorganisms, resulting in false positive results. Therefore, another fluorescence dye, Rhodamine B is used as it forms fluorescent complex together with free fatty acids. This fluorescent complex can be observed at 350 nm under UV light (Kouker & Jaeger, 1987; Jette & Ziomek, 1994).

2.2.2 Titrimetry

Titrimetry is a reliable lipase characterization method. The pH-stat is able to measure the release of 1 μmol of free fatty acids per minute per mL from substrate under standard conditions. Triolein or olive oil is generally used as substrate. Lipase sample is mixed with toluene and propanol and then titrated to phenolphthalein end point. However, this method is time-consuming and tedious. Besides that, it also

requires a lot of lipase for assay ranging from 0.1 to 1 μg/assay (Ghosh *et al.*, 1996; Rathi *et al.*, 2001).

2.2.3 Spectroscopy

In spectroscopy, *p*-nitrophenyl esters are used as substrate. Generally this substrate is used to measure esterase activity but not lipase activity. Lipase activity can be measured using *p*-nitrophenyl palmitate. This *p*-nitrophenol is released when lipase acts on the ester and measured at a wavelength of 410 nm. However, this method cannot be used to measure when lipase sample in acidic condition. Besides, *p*-nitrophenol has different absorption coefficients at different pH values. Another substrate used in this method is β-naphthyl caprylate. Hydrolysis of this colorless substrate forms colored β-naphthol and can be measured at 560 nm using spectrophotometer. This colorimetric assay also uses α-naphthyl esters as substrates (Lanz & Williams, 1973; Kademi *et al.*, 2000). Precipitation of fatty acids released is also measured using spectrophotometer at 500 nm after lipase sample is hydrolyzed by Tween 20 with calcium chloride (Tigerstrom & Stelmaschuk, 1989).

2.2.4 Fluorescence assay

Fluorescent assay measures the release of fluorescent fatty acids. According to Wilton, alkyl groups of triacylglycerols are substituted by fluorescent group (Wilton, 1990). Besides that, 4-methylumbelliferyl oleate (non-fluorescent substrate) is also used in this assay. Fluorescent 4-methylumbelliferone is released by the non-fluorescent substrates after being hydrolyzed by lipase (Jacks & Kircher, 1967).

2.2.5 Chromatography

There are several chromatography methods that can be used to determine the release of fatty acids after lipid substrate is hydrolyzed and in turn measure lipase activity. Thin layer chromatography (TLC) is used to quantify released free fatty acids using densitometric method with radiolabelled triacylglycerols but this sensitive method is very time-consuming (Ruiz & Ochoa, 1997). In gas chromatography (GC), fatty acids are converted to methyl esters and quantified using GC (Bereuter & Lorbeer, 1995). High-performance liquid chromatography (HPLC) can also be used to quantify lipase products (Brune *et al.*, 1992).

2.2.6 Immunological methods

This method involves enzyme-linked immuno assay (ELISA). It is sensitive and can be easily carried out. However, this method is not a suitable routine assay as it requires purified enzyme and antibodies (Doolittle & Ben-Zeev, 1999).

2.2.7 Interfacial pressure

In this study, the assay involves measurement of lipase activity at the interface. One of the measurement techniques is monomolecular film technique which is very sensitive. However, this method is not common because it requires extensive set-ups and a lot of lipase as pH-stat method (Verger, 1980). Another technique is the oil-drop method. Lipase hydrolysis change the surface tension of lipid monolayer and this can be detected by oil-drop method (Nury *et al.*, 1991). This method relies on the shape of oil on the syringe and its shape is correlated to interfacial tension. The shape

of the oil drop changes to pear-form as lipase hydrolyzes the substrate and causes decrease in interfacial tension. This method is able to detect lipase action at a closely controlled interface but contaminants such as free fatty acids can easily disrupt the interfacial tension.

2.2.8 Infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is used to identify the presence of fatty acid esters and free fatty acids with adsorption peaks observed at 1751 and 1715 cm^{-1} respectively. Vegetable oil is usually used for this analysis (Walde & Luisi, 1989).

2.2.9 Densitometry assay using polyhydroxyalkanoate-based microassay

P(3HB-*co*-4HB) film is used as lipase substrate because 4HB can be degraded by lipase. It is one of PHA copolymers. Opaque spot forms on the film when there is lipase activity. The density of the opaque spot is analyzed using free-downloaded software known as ImageJ and its density indicates lipase activity on the film. The density of the spot increases when the lipase activity is getting higher (Ch'ng & Sudesh, 2013).

Table 2.2 Assays for the determination of lipase activity (Gupta *et al.*, 2003)

Assay and substrate	Product analyzed	Principle involved	Remarks
Plate assay			
Tributyrin, acylglycerols and esters of long-chain fatty acids	Short-chain fatty acids	Halo-based or color change of Phenol Red/Victoria Blue/Nile Blue Sulphate, or measurement of fluorescence after complexation of fatty acid with fluorescent dye Rhodamine B.	Convenient for rapid screening.
Titrimetry			
Fats and oils, triacylglycerols, methyl esters	Fatty acids	Neutralization reaction either directly by pH-stat or by pH indicator.	Most reliable and commonly used procedure.
Spectrophotometry			
Fatty acid conjugates of *β*-naphthol	*β*-naphthol	Estimation of *β*-naphthol by complexation with Fast Blue BB.	The ester is not stable at extreme pH.
p-Nitrophenyl esters	*p*-Nitrophenol	Colored product measured at 410 nm.	Convenient method. Preferred during purification procedures. Disadvantage of undergoing spontaneous hydrolysis.
Tweens	Fatty acid	Precipitation of fatty acid with calcium or copper and measurement of turbidity.	Simple, reproducible and sensitive; can be used for quantitative

			assays but often used for plate assays.
Fluorescence assay			
Triacylglycerols with alkyl group substituted with a fluorescent group, e.g. conjugated pyrenyl group	Fluorescent free phenyl groups Shift in fluorescence wavelength after triacylglycerol hydrolysis.	Shift in fluorescence wavelength after triacylglycerol hydrolysis.	Rapid assay, but expensive substrate limits its usage.
Non-fluorescent 4-methylumbelliferyl oleate	Fluorescent 4-methylumbelliferone	Product is analyzed, as it is fluorescent	
Chromatographic procedures (TLC/GC/HPLC)			
Triacylglycerols, fats and oils	Fatty acids	Analysis and quantification of the product or residual substrate through specific columns.	Use depends upon availability of the instrument. Time-consuming for routine analysis, but often recommended for substrate-specificity determination.
Interfacial pressure : monolayer method			
Lipid	Fatty acids	Change in surface pressure due to breakdown of triacylglycerol.	Highly sensitive. Elaborate and extensive set-ups required for accurate estimation.
Interfacial pressure : oil-drop method			
Lipid	Fatty acids	Oil-drop shape is monitored; changes from	Extensive set-ups are required.

		apple to pear shape upon hydrolysis.	
Interfacial pressure : atomic force microscopy			
Lipid bilayers	Fatty acids	Regions of bilayers hydrolyzed by lipases showing deep defects are detected by the atomic-force-microscopy tip.	Provided the first nano-scale picture of kinetics of lipid degradation by lipases. Sophisticated instruments involved.
IR spectroscopy			
Vegetable oils, trioctanoylglycerol	Fatty acid esters and free fatty acids	Lipolysis monitored by recording the Fourier-transform IR spectrum of the entire reaction mixture.	Expensive and sophisticated instruments involved.

2.3 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoate (PHA) is a bio-polyester and biodegradable thermoplastic. It has similar properties as petroleum-synthesized plastics. This polymer has high molecular weight. It can be produced through biosynthesis of various microbes *via* one-stage, two-stage or three-stage cultivation process in excess of renewable carbon source and limited nitrogen, magnesium, oxygen, phosphorus, sulfur etc. The polymer is stored as inclusion or intracellular granule in microbe's cytoplasm as carbon and energy sources. These granules are large and can be observed under phase-contrast light microscopy. This polymer is degradable by most microbes through enzymatic and ultimate degradation.

Homopolymer poly-3-hydroxybutyrate [P(3HB)] was first discovered in year 1925 by Maurice Lemoigne, a microbiologist in Paris (Lemoigne, 1926) as reviewed by Philip and his colleagues (Philip *et al.*, 2007). The physical properties, biosynthesis and biodegradation mechanisms of this polymer have been investigated by many researchers. This polymer has a melting point of approximately 180 ℃ and can be produced *via* three enzymatic reactions. Two acetyl-CoA are condensed to acetoacetyl-CoA *via* 3-ketoacyl-CoA thiolase (PhaA) and reduced to 3-hydroxybutyryl-CoA by NADH- or NADPH-dependent acetoacetyl-CoA reductase (PhaB) followed by polymerization of PHB by PHA synthase (PhaC) from 3-hydroxybutyryl-CoA. Besides homopolymer P(3HB), there are other 3-hydroxyacids that were also discovered. Wallen and Rohwedder had indicated copolymer that consist not only 3HB but also 3-hydroxyvalerate (3HV) (Wallen & Rohwedder, 1974). They found that this copolymer has a lower melting point compared to that of P(3HB). Besides that, analogues of 3-hydroxyacids such as 3-hydroxyheptanoate, 3-hydroxyoctanoates and some other 3-hydroxyacids were first detected from polymer produced by *Bacillus megaterium* (Findlay & White, 1983). PHAs with C_4, C_6 and C_8 were detected as well in sewage sludge (Odham *et al.*, 1986). The presence of poly(3-hydroxyoctatonate) was observed in *Pseudomonas oleovorans* (De Smet *et al.*, 1983) and subsequent study was conducted. The bacterium was fed by alkane, alkanoate and alcohol with different carbon number as carbon source, different composition of 3-hydroxyacid monomers including from C_4 to C_{12} were obtained (Gross *et al.*, 1989; Haywood *et al.*, 1989; Lageveen *et al.*, 1988). There is also a report that showed the ability of *P. aeruginosa* and other *Pseudomonas* species to produce PHAs with 3-hydroxydecanoate by feeding only on gluconate (Timm & Steinbuchel, 1990). Besides that, *Rhodospirillum rubrum*, a photosynthetic bacterium,

was reported to be capable of producing PHAs with C_4 to C_6 monomers after feeding with alkanoic acid (Brandl *et al.*, 1989).

Due to its brittle and inelastic properties, the applications of P(3HB) are limited. In order to overcome these setbacks, additional monomers are incorporated into the polymer to form copolymer such as poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate [P(3HB-*co*-3HV)], poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate [P(3HB-*co*-3HHx)] and poly-3-hydroxybutyrate-*co*-4-hydroxybutyrate [P(3HB-*co*-4HB)]. P(3HB-*co*-3HV) composed of various compositions of 3HV have melting temperature ranging from 75 to 170 ℃, lower than that of P(3HB) (Asrar & Gruys, 2002). Besides that, P(3HB-*co*-3HHx) also has lower melting point and crystallinity compared to that of P(3HB) (Xie & Chen, 2008).

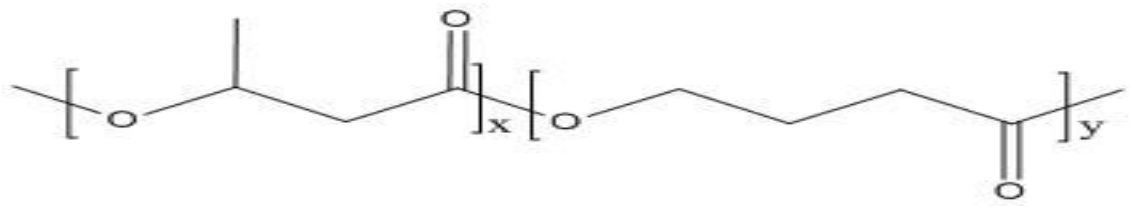
2.3.1 Types of PHA

As mentioned in Section 2.3, there are various types of PHA and they can be classified into two groups, namely natural occurring PHA and non-natural occurring PHA. Natural occurring PHA is the accumulation of PHA by microbes in environment while non-natural occurring PHA is obtained from the addition of precursor or structurally-related carbon source as feeding material under specific cultured conditions (Sudesh & Abe, 2010). There are two types of PHA based on their monomer numbers. Short chain length (scl) PHA is composed of 3 to 5 carbon atoms while medium chain length (mcl) is composed of 6 to 14 carbon atoms. This polymer can be made up by polymerization of similar type of monomer (homopolymer) and even different types of monomers (copolymer) (Nomura *et al.*, 2004). Up to date, there are more than 150 types of monomers that have been

discovered including 3HB, 3HV, 4HB, 3HHx etc. Most of these monomers cannot be naturally synthesized but require structurally-related carbon source or precursor and cultured in suitable conditions. P(3HB-*co*-4HB) is one of PHA copolymers as mentioned in section 2.2.9.

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

P(3HB-*co*-4HB) is made up of 3HB and 4HB monomers as shown in Figure 2.3. It was first discovered by Doi and his colleagues in 1988, which was produced by *C. necator* using 4-hydroxybutyric acid (4HBA) or ɣ-butyrolactone (ɣ-BL) (Doi *et al.*, 1988). This copolymer can be produced by three methods, namely, (i) biosynthesis using mixtures of two carbon sources (Saito & Doi, 1994), (ii) one structurally-related carbon source or precursor (Doi, 1990), (iii) recombinant *Escherichia coli* using only glucose (Valentin & Dennis, 1997). Several microorganisms such as *Delftia acidovorans*, *C. necator*, *Alcaligenes latus*, *Comamonas testosteronii*, *Hydrogenophaga pseudoflava* and *Cupriavidus* sp. are commonly used for biosynthesis of P(3HB-*co*-4HB) by the addition of suitable precursors into the culture medium such as 4HBA, 1,4-butanediol (1,4-BD) and ɣ-BL.



3HB 4HB

Figure 2.3 P(3HB-*co*-4HB) with random distribution of 3HB and 4HB units

The biosynthetic pathway of P(3HB-*co*-4HB) is as shown in Figure 2.2. In this pathway, *C. necator* is used as a model with different carbon sources. When 4HBA is used as carbon source, 4-hydroxybutyryl-coenzyme A (4HB-CoA) is formed. Some of the 4HB-CoA is then metabolized into (*R*)-3-hydroxybutyryl-CoA (3HB-CoA). Copolymerization of 4HB-CoA and 3HB-CoA produces P(3HB-*co*-4HB). Alternatively, oxidation is required for it to be converted to 4-hydroxybutyryl-CoA in the presence of 1,4-BD or 1,6-hexanediol as carbon source. By changing the carbon source to ɣ-BL, 4HB-CoA is formed intracellularly through hydrolysis. A combination of fructose and ɣ-BL as carbon sources produces 3HB-CoA but decreases the 4HB content. Intermediates are obtained *via* β-oxidation when butyric acid and ɣ-BL are used as carbon source. The intermediates inhibit the conversion of 4HB-CoA into 3HB-CoA, increasing the 4HB content (Doi, 1990). *D. acidovorans* is expected to produce P(3HB-*co*-4HB) through similar pathway. The properties and applications of P(3HB-*co*-4HB) are also described in section 2.4.1 and 2.4.2.

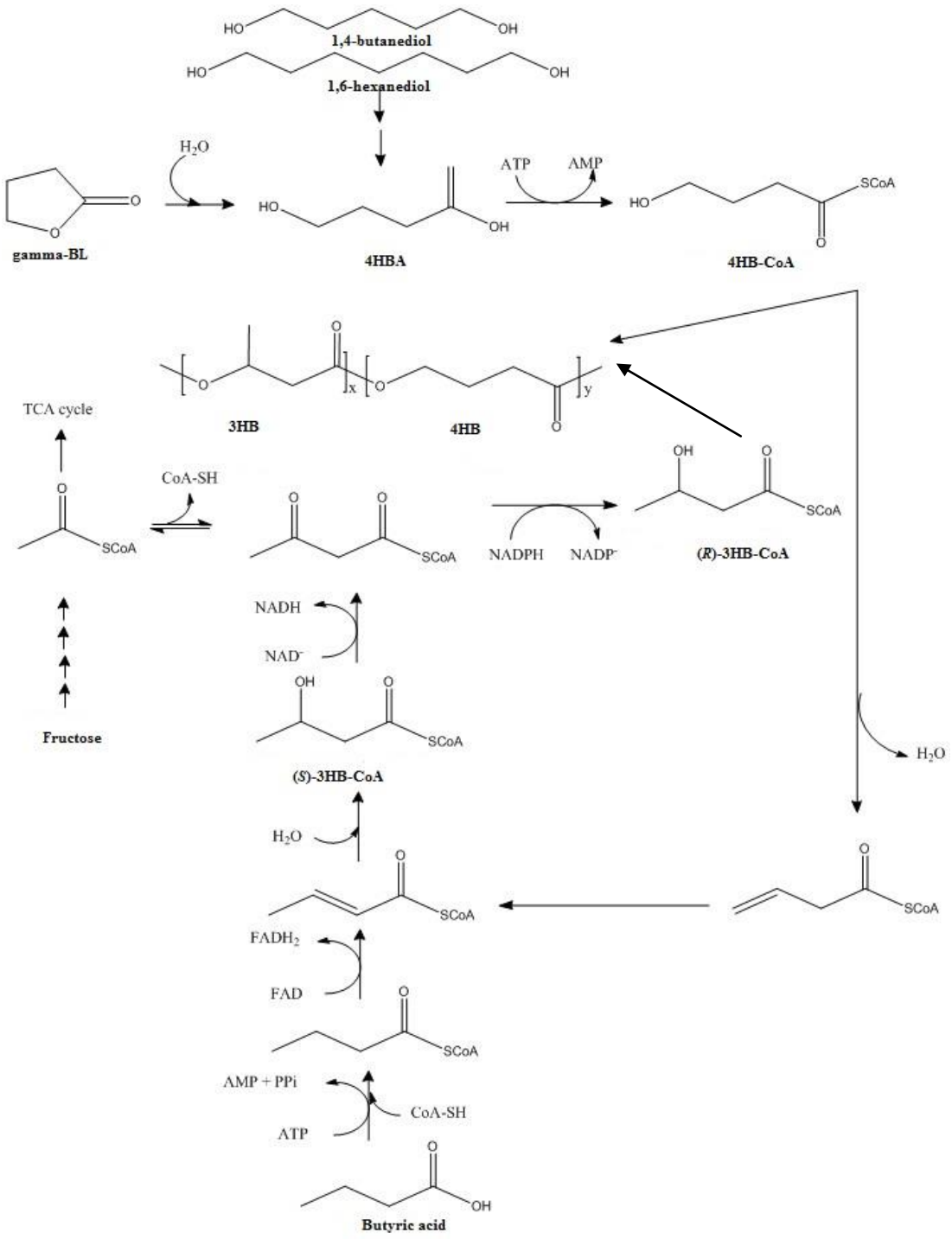


Figure 2.4 Biosynthetic pathway of P(3HB-*co*-4HB) in *C. necator* (Doi, 1990).

2.4.1 Properties of P(3HB-*co*-4HB)

P(3HB-*co*-4HB) is a bioabsorbable and biocompatible polyester because it can be degraded by lipase. The variation of 4HB composition results in different crystallinity of the polymer from crystalline at low composition of 4HB to elastic at high composition of 4HB. The presence of 4HB reduces the stiffness of polymer and the polymer becomes more flexible compared with P(3HB). Small amount of 4HB does not influence much on the crystallinity of P(3HB-*co*-4HB) but the crystallinity of polymer decreases if the % 4HB composition is above 64 %. P(3HB-*co*-4HB) with various 4HB compositions are found to have lower melting point (ranging from 50 to 178 ℃) compared to that of P(3HB) (Jaeger *et al.*, 1995; Saito *et al.*, 1996). The physical and thermal properties of P(3HB-*co*-4HB) are as shown in Table 2.3.

Table 2.3 Physical and thermal properties of P(3HB-*co*-4HB) (Saito *et al.*, 1996)

	4HB fraction (mol %)										
	0	3	7	10	16	27	64	78	82	90	100
Melting temp (℃)	178		172		130		50	49	52	50	53
Glass transition temp (℃)	4		−2		−7		−35	−37	−39	−42	−48
Crystallinity (%)	60	55	50	45	45	40	15	17	18	28	34
Density (g/cm^3)	1.250			1.232	1.234	1.234					
Water uptake (wt %)	0.32			0.20	0.14	0.45					
Stress at yield (MPa)		34		28	19						14
Elongation at yield (%)		4		5	7						17
Tensile strength (MPa)	43	28		24	26		17	42	58	65	104
Elongation to break (%)	5	45		242	444		591	1120	1320	1080	1000