BIOSYNTHESIS OF POLYHYDROXYALKANOATE BIOPLASTIC FROM GLYCEROL BY ENGINEERED ESCHERICHIA COLI

CHITWADEE PHITHAKROTCHANAKOON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (MOLECULAR GENETICS AND GENETIC ENGINEERING) FACULTY OF GRADUATE STUDIES MAHIDOL UNIVERSITY 2014

COPYRIGHT OF MAHIDOL UNIVERSITY

Thesis entitled **BIOSYNTHESIS OF POLYHYDROXYALKANOATE BIOPLASTIC FROM GLYCEROL BY ENGINEERED** ESCHERICHIA COLI

..... Ms. Chitwadee Phithakrotchanakoon Candidate

..... Asst. Prof. Kusol Pootanakit, Ph.D. Major advisor

..... Mr. Verawat Champreda, Ph.D. Co-advisor

..... Asst. Prof. Sutipa Tanapongpipat, Ph.D. Co-advisor

Assoc. Prof. Apinunt Udomkit, Ph.D. Co-advisor

..... Assoc. Prof. Wipa Chungjatupornchai, Ph.D. Co-advisor

..... Prof. Banchong Mahaisavariya, Assoc. Prof. Apinunt Udomkit, Ph.D. M.D., Dip Thai Board of Orthopedics **Program Director** Doctor of Philosophy Program in Dean Molecular Genetics and Genetic Faculty of Graduate Studies Mahidol University Engineering Institute of Molecular Biosciences Mahidol University

Thesis entitled BIOSYNTHESIS OF POLYHYDROXYALKANOATE BIOPLASTIC FROM GLYCEROL BY ENGINEERED ESCHERICHIA COLI

was submitted to the Faculty of Graduate Studies, Mahidol University for the degree of Doctor of Philosophy (Molecular Genetics and Genetic Engineering)

on

July 31, 2014

	Ms. Chitwadee Phithakrotchanakoon Candidate
	Assoc. Prof. Panadda Boonserm, Ph.D. Chair
Mr. Verawat Champreda, Ph.D.	Asst. Prof. Kusol Pootanakit, Ph.D.
Member	Member
Asst. Prof. Sutipa Tanapongpipat, Ph.D.	Assoc. Prof. Apinunt Udomkit, Ph.D.
Member	Member
Assoc. Prof. Arinthip Thamchaipenet,	Assoc. Prof. Wipa Chungjatupornchai,
Ph.D.	Ph.D.
Member	Member
Prof. Banchong Mahaisavariya, M.D.,Dip Thai Board of Orthopedics Dean Faculty of Graduate Studies Mahidol University	Prof. Prasert Auewarakul, M.D., Dr. med. Director Institute of Molecular Biosciences Mahidol University

ACKNOWLEDGEMENTS

This thesis would not be finished without help of the following thankworthy people. I would like to express my deepest gratitude to my major-advisor, Asst. Prof. Kusol Pootanakit and my co-advisors, Asst. Prof. Sutipa Tanapongpipat and Dr. Verawat Champreda for generous supervision, teaching me to think scientifically and extensive support throughout this thesis project. I am deeply grateful to Assoc. Prof. Wipa Chungjatupornchai and Assoc. Prof. Apinunt Udomkit for their valuable discussion as well as constructive suggestions. Furthermore, I would like to thank my external committee, Assoc. Prof. Arinthip Thamchaipenet who gave valuable suggestions for this thesis.

My appreciation also extends to Prof. Seiichi Aiba, Asst. Prof. Suchada Chanprateep, and Dr. Pattama Pittayakhajonwut for their suggestion in polymer extraction and PHA properties characterization. I am also grateful to Asst. Prof. Sarin Chimnaronk for his kindness in providing plasmid vectors and suggestions in plasmid construction. Further thanks go to Dr. Nipon Pisutpaisal and Dr. Sittiruk Roytrakul for their guidance on the high performance liquid chromatography technique. In addition, I would like to thank Dr. Pattanop Kanokrattana for his suggestion and sincere encouragement. My special thanks are extended to all my friends, all staffs of the Institute of Molecular Biosciences, all staffs of the National Center for Genetic Engineering and Biotechnology (BIOTEC), especially to members of Microbial Cell Factory and Enzyme Technology laboratory for their generosity, helpfulness and friendship. Finally, I would like to express my deepest appreciation and gratefulness to my family for their entire love, care and encouragement.

I am extremely thankful to the scholarship from the Royal Golden Jubilee Ph.D. program for the financial support (PHD/0268/2549).

Chitwadee Phithakrotchanakoon

BIOSYNTHESIS OF POLYHYDROXYALKANOATE BIOPLASTIC FROM GLYCEROL BY ENGINEERED *ESCHERICHIA COLI*

CHITWADEE PHITHAKROTCHANAKOON 5137026 MBMG/D

Ph.D. (MOLECULAR GENETICS AND GENETIC ENGINEERING)

THESIS ADVISORY COMMITTEE : KUSOL POOTANAKIT, Ph.D., SUTIPA TANAPONGPIPAT, Ph.D., VERAWAT CHAMPREDA, Ph.D., WIPA CHUNGJATUPORNCHAI, Ph.D., APINUNT UDOMKIT, Ph.D.

ABSTRACT

Polyhydroxyalkanoates (PHAs) are potentially used as bio-plastics. However, the high cost of PHAs limits their use in the commodity market. Crude glycerol is an alternative carbon source for PHAs production. Engineered Escherichia coli strains were constructed to investigate their ability to synthesize poly(3-hydroxybutyrate) [P(3HB)] and Short-Chain-Length-co-Medium-Chain-Length PHAs (SCL-MCL-PHAs) from pure and crude glycerol. PHA biosynthesis-related genes: B-ketothiolase (phaA), acetoacetyl-CoA reductase (phaB) and PHA synthase (phaC), were cloned into pETDuet-ABCs and co-expressed in E. coli for P(3HB) biosynthesis. The phaA and phaB genes were derived from Ralstonia eutropha whereas 3 different phaCs from 3 different bacteria (Aeromonas hydrophila, R. eutropha and *Pseudomonas putida*) were used. The results showed that the amount of P(3HB) is affected by the type of PhaCs (30 wt%, 24 wt%, and 2 wt% of cell dry weight (CDW), respectively). The stage of cell and the carbon: nitrogen ratio were also found to be the key parameters influencing the amount of P(3HB). To produce SCL-MCL-PHAs, three (R)-specific enoyl-CoA hydratase genes from P. putida (phaJ1_{Pp} and phaJ4_{Pp}) and from A. hydrophila (phaJ_{Ah}) were cloned into pCDFDuet-Js and co-transformed with pETDuet-ABCs. Co-expression of PhaAB with each of PhaCs and PhaJs caused E. coli to produce SCL-MCL-PHAs with different monomer compositions, varying from C4 to C10 from pure glycerol supplemented with dodecanoate. The fractions of MCL-unit were in correlation with the concentration of dodecanoate. When 1% crude glycerol was used as a carbon source, E. coli-ABC_{Ah} produced P(3HB) at 14 wt% of CDW; whereas, E. coli-ABC_{Ah}J_{Ah} produced P(3HB-1 mol% 3HHx) at 3 wt% of CDW when dodecanoate was included in the culture media. The molecular weight of PHAs produced was in the range of 110 to 260 kDa and the thermal properties of P(3HB-co-3HHx) were superior to P(3HB).

KEY WORDS: POLYHYDROXYALKANOATE / GLYCEROL / ESCHERICHIA COLI / COPOLYMER / HYBRID PATHWAY

139 pages

การผลิตพลาสติกชีวภาพชนิด Polyhydroxyalkanoate จากกลีเซอรอล โดย *Escherichia coli* ดัดแปลง

BIOSYNTHESIS OF POLYHYDROXYALKANOATE BIOPLASTIC FROM GLYCEROL BY ENGINEERED *ESCHERICHIA COLI*

จิตวดี พิทักษ์โรจนกุล 5137026 MBMG/D

ปร.ค. (อณูพันธุศาสตร์และพันธุวิศวกรรมศาสตร์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: กุศล ภูธนกิจ, Ph.D., สุทิพา ธนพงศ์พิพัฒน์, Ph.D., วีระวัฒน์ แช่มปรีดา, Ph.D., วิภา จึงจตุพรชัย, Ph.D., อภินันท์ อุดมกิจ, Ph.D.

บทคัดย่อ

Polyhydroxyalkanoates (PHAs) มีศักยภาพที่จะใช้เป็นพลาสติกชีวภาพ อย่างไรก็ตามตลาดการใช้ ้งานของ PHAs ยังถูกจำกัดด้วยราคาที่สูง กลีเซอรอลดิบคือแหล่งคาร์บอนทางเลือกสำหรับการผลิต PHAs ด้คแปลงหลายสายพันธุ์จึงถูกสร้างขึ้นเพื่อศึกษาความสามารถในการผลิต coli Escherichia PHAs) จากกลีเซอรอลบริสุทธิ์และกลีเซอรอลคิบ โคยยีนที่เกี่ยวข้องกับการผลิต PHA ใค้แก่ B-ketothiolase (phaA) acetoacetyl-CoA reductase (phaB) และ PHA synthase (phaC) ถูกโคลนเข้า pETDuet-ABCs และถูก ้เหนี่ยวนำให้แสดงออกร่วมกันใน E. coli เพื่อผลิต P(3HB) โดยที่ยืน phaA และ phaB มาจาก Ralstonia eutropha ในขณะที่ phaCs 3 ชนิค มาจากแบคทีเรีย 3 ชนิคคือ Aeromonas hydrophila R. eutropha และ Pseudomonas putida ผลการทดลองแสดงให้เห็นว่าชนิดของ phaCs ส่งผลต่อปริมาณของ P(3HB) ที่ผลิตได้ (30% 24% และ 2% ้งองน้ำหนักเซลล์แห้งตามลำดับ) ระขะงองเซลล์และสัคส่วนระหว่างการ์บอนและ ในโตรเจนในอาหารเลี้ยงมีผล ต่อปริมาณ P(3HB) เช่นกัน และเพื่อที่จะผลิต SCL-MCL-PHAs จึง โคลนยืน (R)-specific enovl-CoA hydratase 3 ชนิด จาก P. putida (phaJ1_{pn} and phaJ4_{pn}) และ A. hydrophila (phaJ₄) เข้า pCDFDuet-Js และส่งถ่ายพร้อมกับ pETDuet-ABCs พบว่าการแสดงออกร่วมกันของ PhaAB กับแต่ละชนิดของ PhaCs และ PhaJs ทำให้ E. coli ผลิต SCL-MCL-PHAs ที่ประกอบด้วยโมโนเมอร์ที่มีจำนวนการ์บอนตั้งแต่ 4 ถึง 10 อะตอมจากการใช้กลีเซอรอล บริสุทธิ์ร่วมกับโคเคกการ์โนเอต และสัดส่วนของโมโนเมอร์สัมพันธ์กับกวามเข้มข้นของโคเคกการ์โนเอต ็นอกจากนี้เมื่อกลีเซอรอลดิบถูกใช้เป็นแหล่งการ์บอน พบว่า E. coli-ABC 46 ผลิต P(3HB) ได้ 14% ของน้ำหนัก เซลล์แห้ง ในขณะที่ E. coli-ABC (J, J, ผลิต P(3HB-1 mol% 3HHx) ได้ 3% ของน้ำหนักเซลล์แห้งเมื่อมีการเติม ์ โคเคกคาร์ โนเอตลงในอาหารเลี้ยงเชื้อ น้ำหนักโมเลกุลของ PHAs ที่ได้อยู่ในช่วง 110 ถึง 260 กิโลคาร์ลตัน และ P(3HB-co-3HHx) แสดงคุณสมบัติด้านความร้อนที่ดีกว่า P(3HB)

139 หน้า

CONTENTS

					Page
ACKNOWLED	GEMEN	ГS			iii
ABSTRACT (E	NGLISH)			iv
ABSTRACT (T	HAI)				v
LIST OF TABI	LES				xi
LIST OF FIGU	RES				xii
LIST OF ABBE	REVIATIO	ONS			xvi
CHAPTER I	INTRO	DUCTION	I		1
CHAPTER II	OBJEC	ΓΙΥΕ			6
CHAPTER III	LITERA	ATURE R	EVIEW		7
3.1	History o	of polyhydr	oxyalkanoat	es (PHAs) research	7
3.2	Chemical	structure a	and propertie	s of PHAs	7
3.3	PHAs bic	osynthesis			10
	3.3.1	PHAs bio	synthetic par	thways	11
	3.3.2	PHA-pro	ducing bacte	ria	12
		3.3.2.1	Ralstonia e	putropha	14
		3.3.2.2	Pseudomon	as spp.	17
		3.3.2.3	Aeromonas	spp.	17
		3.3.2.4	Recombina	nt Escherichia coli	19
			3.3.2.4.1	SCL-PHAs production	19
			3.3.2.4.2	MCL-PHAs production	20
			3.3.2.4.3	SCL-MCL-PHAs production	22
3.4	Biodegra	dation of P	HAs		23
3.5	Applicati	ons of PHA	As		23
	3.5.1	Industrial	applications		24
	3.5.2	Medical a	applications		24
	3.5.3	Agricultu	ral application	ons	24

			Page
3.6	Glycerol		26
	3.6.1	Glycerol from biodiesel production	26
	3.6.2	Glycerol catabolism in E. coli	28
	3.6.3	Conversion of glycerol to PHAs	29
CHAPTER IV	MATER	IALS	35
4.1	Bacterial	strains	35
	4.1.1	PHA-producing strains	35
	4.1.2	E. coli strains	35
4.2	Plasmid v	rectors	35
4.3	Synthetic	oligonucleotides	39
4.4	Culture m	nedia	39
	4.4.1	Culture media for native PHA-producing bacteria	39
	4.4.2	Culture media for <i>E. coli</i> harboring pTZ57R/T	
		vector (for cloning)	39
	4.4.3	Culture media for E. coli harboring pETDuet-1	
		and/or pCDFDuet-1 vector (for expression)	39
	4.4.4	Culture media for P(3HB) biosynthesis	40
	4.4.5	Culture media for SCL-MCL-PHAs biosynthesis	40
4.5	Chemical		40
4.6	Enzymes		40
4.7	Antibodie	28	41
4.8	Commerc	ial kits	41
CHAPTER V	METHO	DDS	42
5.1	Genomic	DNA extraction	42
5.2	Competer	nt cell preparation	43
5.3	Construct	ion of recombinant plasmids	43
	5.3.1	Construction of pETDuet-ABCs	43

viii

	5.3.2	Construct	tion of pCDI	FDuet-Js	45
5.4	Recombin	nant protei	n expression		51
5.5	Protein an	nalysis			51
	5.5.1	SDS-PAG	GE		51
	5.5.2	Western	blot analysis		53
5.6	Crude gly	cerol char	acterization		53
5.7	PHA proc	duction			54
	5.7.1	Culture n	nedia and co	nditions	54
		5.7.1.1	P(3HB) bio	osynthesis	54
		5.7.1.2	SCL-MCL	-PHAs biosynthesis	54
	5.7.2	Analytica	al methods		55
		5.7.2.1	Fluorescen	t dye staining	55
		5.7.2.2	High perfor	rmance liquid chromatography	55
			5.7.2.2.1	P(3HB) quantification	55
			5.7.2.2.2	SCL-MCL-PHAs	
				quantification and	
				compositions analysis	55
			5.7.2.2.3	Glycerol quantification	56
		5.7.2.3	PHAs extra	action and purification	56
		5.7.2.4	Nuclear ma	agnetic resonance	58
		5.7.2.5	Gel perme	ation chromatography	58
		5.7.2.6	Differentia	l scanning calorimetry	58
		5.7.2.7	Thermogra	vimetry analysis	58
CHAPTER VI	RESUL '	ТS			59

			I	Page
6.1	Establish	ment of PI	HA biosynthesis pathway in	
	recombin	ant <i>E. coli</i>		59
	6.1.1	Pathway	design and amino acid alignment	59
	6.1.2	Plasmid o	construction	65
	6.1.3	Protein e	xpression	67
6.2	Using put	re glycerol	as a carbon source for PHAs biosynthesis	74
	6.2.1	P(3HB) b	biosynthesis	74
		6.2.1.1	Effect of different PhaC types on growth	
			and P(3HB) biosynthesis by E. coli-ABCs	74
		6.2.1.2	Effect of glycerol concentration on growth	
			and P(3HB) biosynthesis by <i>E. coli</i> -ABC _{Ah}	78
		6.2.1.3	Effect of carbon and nitrogen source on	
			growth and P(3HB) biosynthesis by	
			$E. \ coli$ -ABC _{Ah}	80
		6.2.1.4	Effect of stage of cell at the induction	
			point on P(3HB) biosynthesis	83
	6.2.2	SCL-MC	L-PHAs biosynthesis	84
		6.2.2.1	Effect of different PhaC and PhaJ types	
			on SCL-MCL-PHAs biosynthesis	84
		6.2.2.2	Effect of cultivation time on the	
			P(3HB-co-3HHx) biosynthesis by	
			$E. \ coli$ -ABC _{Ah} J _{Ah}	90
		6.2.2.3	Effect of dodecanoate concentration on	
			the P(3HB-co-3HHx) biosynthesis by	
			E. $coli$ -ABC _{Ah} J _{Ah}	92

6.3 Using crude glycerol as a carbon source for PHAs biosynthesis 94

			Page
	6.3.1	Characterization of crude glycerol compositions	94
	6.3.2	P(3HB) biosynthesis by <i>E. coli</i> -ABC _{<i>Ah</i>}	97
	6.3.3	P(3HB-co-3HHx) biosynthesis by E. coli-ABC _{Ah} J _{Ah}	101
6.4	PHA prop	perties characterization	102
	6.4.1	Chemical structure analysis by NMR	102
	6.4.2	Molecular weight analysis by GPC	105
	6.4.3	Thermal properties analysis by DSC	105
	6.4.4	Degradation temperature analysis by TGA	105
CHAPTER VII	DISCUS	SION	107
7.1	Establish	ment of PHA biosynthesis pathway in	
	recombin	ant E. coli	107
7.2	Using pu	re glycerol as a carbon source for PHAs biosynthesis	108
7.3	Using cru	de glycerol as a carbon source for PHAs biosynthesis	112
7.4	PHA prop	perties characterization	114
CHAPTER VII	I CONCL	USIONS	116
REFERENCES			118
BIOGRAPHY			137

LIST OF TABLES

Table		Page
3.1	Properties of some PHAs and petroleum based plastic	9
3.2	Overview of bacterial strains used for PHAs production	13
3.3	List of enzymes which have been successfully expressed in recombinant	
	E. coli for PHA biosynthesis from fatty acid metabolism	22
3.4	PHAs production from glycerol	30
5.1	Oligonucleotide primers	48
5.2	Genomic DNA template for target PCR fragment	49
5.3	PCR condition used for amplifying target DNA fragment	50
5.4	Components of SDS-PAGE for protein determination	52
6.1	P(3HB) biosynthesis in recombinant E. coli harboring phaABCs	78
6.2	Effect of glycerol concentration on P(3HB) biosynthesis	80
6.3	PHA copolymers biosynthesis from glycerol and dodecanoate in	
	recombinant E. coli harboring phaABCs and phaABCsJs	89
6.4	Effect of sodium dodecanoate concentration on PHA production	93
6.5	Compositions of crude glycerol	95
6.6	The effect of crude glycerol concentration on P(3HB) biosynthesis in	
	$E. \ coli$ -ABC _{Ah}	98
6.7	PHA biosynthesis in <i>E. coli</i> -ABC _{<i>Ah</i>} J_{Ah} using pure glycerol (PG) and	
	crude glycerol (CG) with or without sodium dodecanoate (C12)	101
6.8	Molecular weight and thermal properties of PHAs	106

LIST OF FIGURES

Figure		Page
1.1	Three metabolic pathways that provide various hydroxyalkanoate	
	(HA) monomers for PHAs biosynthesis	4
1.2	The hybrid pathways for biosynthesis of SCL-MCL-PHA copolymers	
	in recombinant E. coli	5
3.1	General structure of polyhydroxyalkanoates (PHAs) and their	
	examples	9
3.2	PHA granule characteristic	10
3.3	The elements involved in P(3HB) metabolism in R. eutropha H16	15
3.4	The organization of <i>pha</i> genes in three native PHA-producing bacteria	16
3.5	Proposed pathway of P(3HB-co-3HHx) biosynthesis in A. caviae from	
	alkanoic acids or oils	18
3.6	Schematic of various strategies for metabolic engineering	20
3.7	Applications of PHAs	25
3.8	Biodiesel reaction	26
3.9	Flow chart of alkaline catalyzed biodiesel production	27
3.10	Glycerol catabolism in <i>E. coli</i>	28
3.11	Overview of the pathways and products converted from glycerol	29
4.1	A physical map of pTZ57R/T vector	36
4.2	A physical map of pETDuet-1 vector	37
4.3	A physical map of pCDFDuet-1 vector	38
5.1	A schematic representation of overall procedure for construction of	
	pETDuet-ABCs	46
5.2	Physical map of recombinant plasmids constructed in this study	47
5.3	PHA extraction and purification flowchart	57
6.1	Amino acid sequence alignment of PhaCs	61

LIST OF FIGURES (cont.)

Figure		Page
6.2	Amino acid sequence alignment of PhaC from A. hydrophila	62
	ATCC 7966 and from A. caviae	
6.3	Amino acid sequence alignment of PhaC1 and PhaC2 from	
	P. putida KT2440	63
6.4	Amino acid sequence alignment of PhaJs	64
6.5	Amino acid sequence alignment of PhaJ from A. hydrophila	
	ATCC 7966 and from A. caviae	64
6.6	Genomic DNA extracted from wild type PHA-producing bacteria	65
6.7	PCR amplification of β -ketothiolase (<i>phaA</i>), NADPH-dependent	
	acetoacetyl-CoA reductase (phaB), and PHA synthase (phaC) genes	
	from genomic DNA of wild type PHA-producing bacteria	66
6.8	PCR amplification of (<i>R</i>)-specific enoyl-CoA hydratase (<i>phaJ</i>) genes	
	from genomic DNA of wild type PHA-producing bacteria	66
6.9	Heterologous protein expression of β -ketothiolase (PhaA), NADPH-	
	dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase	
	(PhaC) proteins in <i>E. coli</i> BL21(DE3)	68
6.10	SDS-PAGE and western blot analysis of heterologous protein	
	expression of (R)-specific enoyl-CoA hydratase (PhaJ) proteins in	
	E. coli BL21(DE3)	69
6.11	Co-expression of β -ketothiolase (PhaA), NADPH-dependent	
	acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and	
	(<i>R</i>)-specific enoyl-CoA hydratase (PhaJ1 _{<i>Pp</i>}) proteins in <i>E. coli</i>	
	BL21(DE3)	71
6.12	Co-expression of β -ketothiolase (PhaA), NADPH-dependent	
	acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and	
	(<i>R</i>)-specific enoyl-CoA hydratase (PhaJ4 _{<i>Pp</i>}) proteins in <i>E. coli</i>	
	BL21(DE3)	72

LIST OF FIGURES (cont.)

Figure		Page
6.13	Co-expression of β -ketothiolase (PhaA), NADPH-dependent	
	acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and	
	(R)-specific enoyl-CoA hydratase (PhaJ _{Ah}) proteins in E. coli	
	BL21(DE3)	73
6.14	Growth profiles of recombinant E. coli-ABCs strains cultured in	
	minimum medium (M9) containing 2% (v/v) glycerol as a sole carbon	
	source	75
6.15	Protein expression profiles of <i>E. coli</i> -ABC _{<i>Re</i>} , <i>E. coli</i> -ABC _{<i>Ah</i>} , and	
	<i>E. coli</i> -ABC2 _{<i>Pp</i>} analyzed by SDS-PAGE	76
6.16	Nile blue A fluorescent dye staining of P(3HB) granules in	
	recombinant E. coli-ABCs	77
6.17	Nile blue A fluorescent dye staining of P(3HB) granules in	
	recombinant E. coli-ABCs as observed by confocal laser scanning	
	microscope	77
6.18	Effect of glycerol concentration on the growth profiles of	
	$E. \ coli$ -ABC _{Ah}	79
6.19	Effect of carbon and nitrogen source on the growth profiles of	
	$E. \ coli$ -ABC _{Ah}	81
6.20	Effect of carbon and nitrogen source on pH profiles of E. coli-ABC _{Ah}	81
6.21	Effect of carbon and nitrogen source on the P(3HB) biosynthesis	82
6.22	Effect of stage of cell at the induction point on P(3HB) biosynthesis	83
6.23	Protein expression profiles of <i>E. coli</i> -ABC _{<i>Re</i>} J1 _{<i>Pp</i>} , <i>E. coli</i> -ABC _{<i>Ah</i>} J1 _{<i>Pp</i>} ,	
	and <i>E. coli</i> -ABC2 _{<i>Pp</i>} J1 _{<i>Pp</i>} as analyzed by SDS-PAGE	85
6.24	Protein expression profiles of <i>E. coli</i> -ABC _{<i>Re</i>} J4 _{<i>Pp</i>} , <i>E. coli</i> -ABC _{<i>Ah</i>} J4 _{<i>Pp</i>} ,	
	and <i>E. coli</i> -ABC2 _{<i>Pp</i>} J4 _{<i>Pp</i>} as analyzed by SDS-PAGE	86
6.25	Protein expression profiles of <i>E. coli</i> -ABC _{<i>Re</i>} J_{Ah} , <i>E. coli</i> -ABC _{<i>Ah</i>} J_{Ah} ,	
	and <i>E. coli</i> -ABC2 _{<i>Pp</i>} J _{<i>Ah</i>} as analyzed by SDS-PAGE	87

LIST OF FIGURES (cont.)

Figure		Page
6.26	HPLC analysis of P(3HB-6.9 mol% 3HHx)	88
6.27	Time-dependent change in OD600, pH, % PHA content, and	
	mol% 3HHx	91
6.28	The characteristic of crude glycerol produced from biodiesel	
	production as a by-product	95
6.29	Growth profiles of recombinant E. coli cultured in crude glycerol	96
6.30	SDS-PAGE analysis of protein expression levels in $E. \ coli$ -ABC _{Ah}	
	cultured in crude glycerol	99
6.31	Densitometry analysis of protein band intensity	100
6.32	Chemical structure analysis of P(3HB) and P(3HB-3 mol% 3HHx)	
	by ¹ H NMR	103
6.33	Chemical structure analysis of P(3HB) and P(3HB-3 mol% 3HHx)	
	by ¹³ C NMR	104
6.34	DSC thermogram of PHAs	106

LIST OF ABBRIVIATIONS

DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
g	gram (s)
x g	centrifugal force
h	hour (s)
HPLC	high performance liquid chromatography
IPTG	isopropyl-thio-β-D-galactopyranoside
kb	kilobase (s)
kDa	kilodalton (s)
LB	Luria-Bertani
$M_{ m w}$	weight average molecular weight
MCL-PHA	Medium-Chain-Length polyhydroxyalkanoate
mM	millimolar
mg	milligram (s)
min	minute (s)
mL	milliliter (s)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
ng	nanogram (s)
nm	nanometer (s)
NMR	nuclear magnetic resonance
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDI	polydispersity index
PHA	polyhydroxyalkanoate

LIST OF ABBRIVIATIONS (cont.)

rpm	revolution per minute		
SCL-PHA	Short-Chain-Length polyhydroxyalkanoate		
SDS	sodium dodecyl sulfate		
T _d	degradation temperature		
$T_{ m g}$	glass transition temperature		
$T_{ m m}$	melting temperature		
TEMED	N,N,N',N'-tetramethyl-ethylenediamine		
TGA	thermogravimetric analysis		
μg	microgram (s)		
μL	microliter (s)		
v/v	volume/volume		
w/v	weight/volume		
w/w	weight/weight		

CHAPTER I INTRODUCTION

Nowadays plastic materials are an integral part of our life due to their excellent properties such as ease of molding, light weight, chemical resistance and their durability. Thus, the use of plastics has increased rapidly over the past few decades. Based on the report by Global Industry Analysts, global plastic consumption will reach 297.5 million tons by 2015. However, the accumulation of the plastic wastes has become a critical environmental problem as most conventional plastics are produced from petroleum oil which take several centuries to be degraded. To solve this problem, biodegradable plastic is considered a promising alternative (1-3).

One such compound is polyhydroxyalkanoates (PHAs). PHAs is a family of microbial polyester with potential use as bio-plastic for many applications such as food packaging, drug delivery, medical implants, agriculture/horticulture, automotive and household appliances. PHAs can be produced from a range of renewable carbon substrates. PHAs are produced by some types of bacteria as intracellular granules for carbon and energy storage under unbalance growth conditions in the presence of excess carbon source (4). Unlike the petroleum-based plastics, PHAs are biodegradable, thus reducing waste accumulation and greenhouse gas emission. Moreover, environmental impact analysis shows that life cycle of bio-based plastics are superior to petroleum-based plastic (5).

Depending on the monomer structure, PHAs can be classified into three groups which are Short-Chain-Length PHAs (SCL-PHAs) containing 3 to 5 carbon atoms in the monomer, Medium-Chain-Length PHAs (MCL-PHAs) containing 6 to 14 carbon atoms in the monomer and SCL-MCL-PHA copolymer containing both SCL-and MCL-monomers (6). The difference in monomer compositions affects the properties of PHAs. SCL-PHAs such as poly(3-hydroxybutyrate) or P(3HB) have high crystallinity. MCL-PHAs, on the other hand, have high flexibility. SCL-MCL-PHA SCL-MCL-PHA copolymers range from high crystallinity to elasticity, depending on the molar fraction of the various monomer constituents. For example, a copolymer composed of a high fraction of 3HB and a low fraction of 3-hydroxyhexanoate (3HHx) possesses properties similar to low-density polyethylene (7).

In nature, only a few bacteria are known to produce SCL-MCL-PHA such as Pseudomonas sp. 61-3, which produces a random copolymer consisting of C4 to C12 monomer units from gluconate (8), Aeromonas caviae and Aeromonas hydrophila, which produce P(3HB-co-3HHx) from alkanoic acids (9, 10). Unfortunately, Aeromonas spp. have been reported as pathogens (11). Therefore, they are not practical to use for large scale production. Moreover, it is difficult to adjust the content and composition of copolymers produced by these bacteria, as their metabolic pathways are poorly characterized and no tools for genetic modification are available. A more practical approach to the production of copolymers with specific properties for industrial application is genetic engineering of model microorganisms (e.g., Escherichia coli) that do not naturally produce PHAs.

There are three well-known pathways for PHAs biosynthesis in bacteria (Fig 1.1) (12). Pathway I is found in many bacteria and has been extensively studied in *Ralstonia eutropha*, which produces SCL-PHA. This species produces SCL-PHA through the action of β -ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) enzymes. PhaA and PhaB generate the SCL-monomer: (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] by sequentially catalyzing acetyl-CoA to acetoacetyl-CoA and to (*R*)-3HB-CoA respectively. PhaC then polymerizes (*R*)-3HB-CoA monomers to the P(3HB) polymer. Pathways II and III are found mostly in the *Pseudomonas* bacteria, and are mainly involved in the production of MCL-PHAs. They are linked to the fatty acid β -oxidation and fatty acid biosynthesis pathway *via* (*R*)-specific enoyl-CoA hydratase (PhaJ) and (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) enzymes, respectively.

The production of SCL-MCL-PHA copolymers in both wild type and recombinant strains has been previously investigated (13-16). However, the use of PHAs as commercialized material is still limited. This is because of their high production cost. For example the market price of P(3HB) from Biomer (Germany) is $\in 12/kg$ while the market price of polypropylene is only $\in 0.74/kg$ (1). According to

Choi and Sang (1997), the price of PHAs mainly depends on substrate cost which accounts for 40-48% of the total production cost (17). Therefore, one approach to reduce the production cost is by using economical substrates such as waste materials as alternative carbon source. Many waste materials have been studied for use as alternative substrates for PHA production (1). Recently, glycerol, a by-product from the growing biodiesel industry, is becoming a promising inexpensive carbon source (18). Thus it is interesting to develop the bio-production system for SCL-MCL-PHA from crude glycerol to reduce the production cost of PHAs.

In this study, the conversion of glycerol to SCL-MCL-PHA copolymers was investigated. Based on the understanding of the PHA biosynthetic pathway in native strains (12), a hybrid pathway (Fig 1.2) was constructed in E. coli by genetic engineering approach. The pathway contains two monomers supplying routes including β -ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) for providing SCL-monomer: (R)-3HB-CoA and enoyl-CoA hydratase (PhaJ) for providing MCL-monomer. PhaC polymerizes the sythesized monomers to copolymers. Additionally, to study the effects of different pha genes combination on the composition of copolymers, three wild type PHA-producing bacteria, Ralstonia eutropha (R. eutropha), Aeromonas hydrophila (A. hydrophila) and Pseudomonas putida (P. putida), were selected as candidates due to their ability to produce different types of PHAs. PhaA_{Re} and PhaB_{Re} of R. eutropha with three phaC genes which are phaC of R. eutropha (pha C_{Re}), A. hydrophila ATCC 7966 (pha C_{Ah}) and P. putida KT2440 ($phaC2_{Pp}$) and three phaJ genes which are $phaJ_{Ah}$, $phaJ1_{Pp}$ and $phaJ4_{Pp}$ were heterologously co-expressed in recombinant E. coli. Two groups of recombinant plasmids were created. The first group harbors phaA, phaB and phaC genes (pETDuet-ABCs). The second group harbors phaJ genes (pCDFDuet-Js). The ability of recombinant E. coli to synthesize PHAs from glycerol was investigated by culturing the cell in minimum medium containing glycerol as the sole carbon source. The physical and thermal properties of the produced polymers were also characterized. This study provides the basis for cost-effective production of PHA copolymers with properties suitable for industrial application to improve the competitiveness of the bioplastic industry.



Figure 1.1 Three metabolic pathways that provide various hydroxyalkanoate (HA) monomers for PHAs biosynthesis [modified from (12) with permission from Elsevier].

There are three well-known PHA biosynthetic pathways. Pathway I provides SCL-monomer from acetyl-CoA. Pathway II and pathway III provide SCLand MCL-monomers from the intermediates of fatty acid β-oxidation and fatty acid biosynthesis pathway, respectively. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaJ, (*R*)-specific enoyl-CoA hydratase; PhaG, 3-hydoxyacyl-ACP-CoA transferase; FadA, 3-ketoacyl-CoA thiolase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadE, acyl-CoA dehydrogenase; FabB, 3-ketoacyl-ACP synthase I; FabG, 3-ketoacyl-ACP reductase; FabI, enoyl-ACP reductase; FabZ, 3-hydroxyacyl-ACP dehydratase.

Ph.D.(Molecular Genetics and Genetic Engineering) / 5



Figure 1.2 The hybrid pathways for biosynthesis of SCL-MCL-PHA copolymers in recombinant *E. coli* [reproduced from (19) with permission from JSBA].

The hybrid pathway was designed by combining two monomer-supplying pathways with one polymerization pathway. Glycerol was used as a carbon source for generating (*R*)-3-Hydroxybutyryl-CoA (SCL-monomer) by the activities of β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), whereas fatty acid was used as a carbon source for generating (*R*)-3-Hydroxyacyl-CoA (MCL-monomer) by the activity of (*R*)-specific enoyl-CoA hydratase (PhaJ). The final step is polymerization by PHA synthase (PhaC) to biosynthesize SCL-MCL-PHA.

CHAPTER II OBJECTIVES

1. To establish the pathway for biosynthesis of SCL-PHA and SCL-MCL-PHA copolymers in recombinant *E. coli*.

2. To investigate the effect of the substrate specificity of three different PHA synthase enzymes and three different (R)-specific enoyl-CoA hydratase enzymes for PHA biosynthesis.

3. To investigate the types and content of PHAs produced by recombinant *E. coli* growing under pure or crude glycerol as carbon substrate and to investigate the effect of fatty acids using as co-substrate.

4. To investigate the physical and thermal properties of PHAs produced by recombinant *E. coli*.

CHAPTER III LITERATURE REVIEW

3.1 History of polyhydroxyalkanoates (PHAs) research

In 1926, poly(3-hydroxybutyrate) [P(3HB)] was firstly discovered and extracted from *Bacillus megaterium* by Lemoigne (20). But further research on P(3HB) failed to spread because of the limited methods for detection and characterization. The accumulated P(3HB) granule was later proven at the end of 1950s that it was an intracellular storage granule for carbon and energy (21, 22). Twenty years later, PHAs containing 3-hydroxyalkanoic acid (3HA) other than 3-hydroxybutyric acid (3HB) were identified (23, 24). This finding encouraged the research on PHAs. Numerous other 3HA constituents such as 3-hydroxyvaleric acid (3HV), 3-hydroxyhexanoic acid (3HHx), 3-hydroxyheptanoic acid (3HHp) and 3-hydroxyoctanoic acid (3HO) were discovered by the end of 1980s (25, 26). At the same time, the genes encoding for the enzymes involved in PHA biosynthesis from *Ralstonia eutropha* were cloned and the key enzyme for polymerization was named as PHA synthase (27, 28). To date, about 59 PHA synthase enzymes have been identified (29).

3.2 Chemical structure and properties of PHAs

PHAs are a family of linear polyester composed of several units of hydroxyalkanoate. The general chemical structure of PHAs is shown in Figure 3.1, where R is alkyl side chain which varies from C0 to C11 and n is number of monomer units in each polymer chain which varies from 100 to 30,000. Moreover, the monomers which contain hydroxyl groups at positions 4, 5 or 6 and the side chain group containing unsaturations or substitutions are also found. About 150 different monomer constituents of PHAs have been reported (29). Generally, PHAs can be

classified into three major groups, depending on their monomer structures. Short-Chain-Length PHAs (SCL-PHAs) compose of monomers containing 3 to 5 carbon atoms, for example, poly(3-hydroxybutyrate) P(3HB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]. Medium-Chain-Length PHAs (MCL-PHAs) compose of monomers containing 6 to 14 carbon atoms, for example, poly(3-hydroxyoctanoate) P(3HO) and poly(3-hydroxyoctanoate-*co*-3-hydroxydecanoate) [P(3HO-*co*-3HD)]. Short-Chain-Length-*co*-Medium-Chain-Length PHAs (SCL-MCL-PHAs) contain both SCL-monomers and MCL-monomers for example, Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)].

The differences in monomer compositions affect the properties of PHAs. SCL-PHAs have high crystallinity whereas MCL-PHAs have higher flexibility (30). Therefore, the properties of SCL-MCL-PHAs can range from high crystallinity to elasticity, depending on the molar fraction of various monomer constituents. These SCL-MCL-PHAs possess superior material properties compared to those of SCL-PHAs and MCL-PHAs (16, 31). Moreover, it was found that copolymer: P(HB-*co*-HHx) has better biocompatibility than P(3HB) homopolymer (32). Due to these highly diverse PHA-monomer constituents, PHAs can be used in many applications comparable to petroleum based plastic. Table 3.1 compares the properties of PHAs to petroleum-based plastics (4, 12, 33).

Fac. of Grad. Studies, Mahidol Univ.

Ph.D.(Molecular Genetics and Genetic Engineering) / 9

	R group	Type of PHA	PHA name
$-\left(\begin{matrix} R & O \\ -CH-CH_2-C \\ n \end{matrix}\right)$	-CH ₃	SCL-PHA	Poly(3-hydroxybutyrate)
	$-C_{3}H_{7}$	MCL-PHA	Poly(3-hydroxyhexanoate)
	-CH ₃ , -C3H ₇	SCL-MCL-PHA	Poly(3-hydroxybutyrate-co-
			3-hydroxyhexanoate)

Figure 3.1 General structure of polyhydroxyalkanoates (PHAs) and their examples.

PHAs are divided into three groups which are SCL-PHAs, MCL-PHAs and SCL-MCL-PHAs based on their monomer structure. The difference in monomer structure depends on the alkyl side chain group (R group). In general, R group varies from C0 to C11 and n is number of monomer units which vary from 100 to 30,000.

Table 3.1 Properties of some PHAs and petroleum based plastic [data from (4, 12, 33)].

Properties	P(3HB)	P(3HB-co-3HHx) (10 mol% 3HHx)	P(3HO-co-3HHx) (11 mol% 3HHx)	РР	LDPE
$T_{\rm m}$ (°C)	177	127	61	176	130
$T_{\rm g}$ (°C)	4	-1	-36	-10	-36
Crystallinity (%)	60	34	30	50-70	20-50
Extension to break (%)	5	400	300	400	620
Tensile strength (Mpa)	43	21	-	38	10

PP, polypropylene; LDPE, low density polyethylene; $T_{\rm m}$, melting temperature; $T_{\rm g}$, glass transition temperature

3.3 PHAs biosynthesis

PHAs can be produced by many types of microorganism from various renewable substrates such as sugar, starch, and plant oil. In most cases, bacteria produce PHAs under unbalance growth conditions such as limitation of nitrogen, phosphorus, or oxygen in the presence of excess carbon. PHAs are accumulated inside the cells as intracellular granules for carbon and energy storage (Fig 3.2) (34, 35). It is an ideal storage granule due to its high molecular weight and low solubility which does not affect the bacteria osmotic pressure (36). Also, bacteria containing PHA granules are able to survive during starvation in the absence of exogeneous carbon source by utilizing the accumulated PHAs (37).



Figure 3.2 PHA granule characteristic. (A) PHA granules accumulated inside *Ralstonia eutropha*. Bars, 0.2 μ m. (B) Cartoon drawing represents the compositions of PHA granule [(A) is reproduced from (35) with permission from Society for General Microbiology and (B) is reproduced from (34) with permission from Nature Publishing Group].

PHA is deposited as spherical intracellular granules with an amorphous hydrophobic core surrounded by proteins involving in PHA metabolism. In PHA-producing bacteria, PHAs are produced when the carbon source is excess while the other nutrients are limited. When the carbon starvation occurs, the reserved PHA granules are mobilized by intracellular PHA depolymerase.

3.3.1 PHA biosynthetic pathways

The constituent of PHAs depends on the carbon sources, PHA synthases, and monomer supplying metabolic routes (38, 39). There are three well-known PHA biosynthesis pathways (Fig 1.1) (12).

Pathway I produces SCL-PHAs. Acetyl-CoA is a central molecule for P(3HB) biosynthesis. Under normal growth condition, acetyl-CoA is oxidized through tricarboxylic acid cycle (TCA) to CO₂. However, under nutrient limitation, the accumulation of acetyl-CoA and NADH, starts P(3HB) biosynthesis (40). Three main catalysis enzymes: β -ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC), are responsible for P(3HB) biosynthesis. Two molecules of acetyl-CoA are condensated to acetoacetyl-CoA by the function of PhaA. Then acetoacetyl-CoA is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA by PhaB. (*R*)-3-hydroxybutyryl-CoA molecules are then be polymerized by PhaC to produce P(3HB).

Pathway II and III produce MCL-PHAs. Pathway II involves fatty acid β-oxidation cycle. When bacteria are grown on fatty acids, fatty acids are catabolized by β-oxidation, providing precursors for MCL-PHAs biosynthesis. β-oxidation of saturated fatty acids has four basic steps. First, fatty acyl-CoA is dehydrogenated by acyl-CoA dehydrogenase (FadE) yielding trans-2-enoyl-CoA. In the second step, double bond of *trans*-2-enoyl-CoA, water is added to the vielding (S)-3-hydroxyacyl-CoA by the activity of enoyl-CoA hydratase (FadB). The third step, (S)-3-hydroxyacyl-CoA is dehydrogenated to form 3-ketoacyl-CoA by the activity of 3-hydroxyacyl-CoA dehydrogenase (FadB). The fourth step, 3-ketoacyl-CoA thiolase (FadA) catalyzes (S)-3-hydroxyacyl-CoA to produce acyl-CoA shortened by two carbon atoms and acetyl-CoA.

Two intermediates including *trans*-2-enoyl-CoA and 3-ketoacyl-CoA seem to be the precursors of PHAs. To channel these two intermediates to PHA biosynthesis, it is necessary to convert them to (R)-3-hydroxyacyl-CoAs which are the monomer substrates of PHA synthase. Two enzymes, (R)-specific enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-ACP reductase (FabG), are responsible for converting *trans*-2-enoyl-CoA and 3-ketoacyl-CoA to (R)-3-hydroxyacyl-CoAs, respectively.

Thus, monomer molecules varying in the carbon atom number are produced by shortening two carbon of acetyl-CoA out of each cycle of β -oxidation (41).

Pathway III involves the fatty acid biosynthesis pathway (*de novo* fatty acid synthesis). There are four steps in fatty acid elongation cycle. Two carbon atoms elongation is started with condensation of acyl-ACP with malonyl-ACP by the activity of 3-ketoacyl-ACP synthase I (FabB) or II (FabF) resulting in 3-ketoacyl-ACP. The second step, 3-ketoacyl-ACP is reduced by 3-ketoacyl-ACP reductase (FabG) yielding (*R*)-3-hydroxyacyl-ACP. The third step, (*R*)-3-hydroxyacyl-ACP is dehydrated by 3-hydroxyacyl-ACP dehydratase (FabZ) yielding enoyl-ACP. The fourth step, enoyl-ACP is reduced by enoyl-ACP reductase (FabI) resulting in acyl-ACP with two added carbon atoms (42). An intermediate: (*R*)-3-hydroxyacyl-ACP seems to be a precursor for PHAs. (*R*)-3-hydroxyacyl-ACP is converted to (*R*)-3hydroxyacyl-ACP-CoA transferase (PhaG) (43). Unlike pathway II, paythway III is considered as a non-substrate related pathway (such as from glucose and gluconate).

3.3.2 PHA-producing bacteria

There are several bacterial strains that can produce PHAs. The overview of bacterial strains used for PHAs production is shown in Table 3.2 (30). Based on the type of PHAs, three native PHA-producing bacterial strains have been studied intensively. These include *Ralstonia eutropha* producing SCL-PHAs, *Aeromonas* strains producing SCL-MCL-PHAs, and *Pseudomonas* strains producing MCL-PHAs. In addition, several recombinant *E. coli* strains have been constructed for PHAs production (28, 44-46).

Table 3.2 Overview of bacterial strains used for PHAs production [reproducedfrom (30) with permission from John Wiley and Sons].

Bacterial strain (s)	Carbon source (s)	Polymer (s) produced	Reference
Aeromonas hydrophila Alcaligenes latus	Lauric acid, oleic acid Malt, soy waste, milk waste, vinegar waste, sesame oil	mcl-PHAs PHB	(Lee <i>et al.</i> 2000; Han <i>et al.</i> 2004) (Wong <i>et al.</i> 2004, 2005)
Bacillus cereus	Glucose, e-caprolactone, sugarbeet molasses	PHB, terpolymer	(Labuzek and Radecka 2001; Yilmaz and Beyatli 2005; Valappil <i>et al.</i> 2007)
Bacillus spp.	Nutrient broth, glucose, alkanoates, &-caprolactone, soy molasses	PHB, PHBV, copolymers	(Katircioglu <i>et al.</i> 2003; Shamala <i>et al.</i> 2003; Tajima <i>et al.</i> 2003; Yilmaz <i>et al.</i> 2005; Full <i>et al.</i> 2006)
Burkholderia sacchari sp. nov.	Adonitol, arabinose, arabitol, cellobiose, fructose, fucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHB∨	(Brämer <i>et al.</i> 2001)
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, PHB∨	(Keenan <i>et al.</i> 2004; Nakas <i>et al.</i> 2004; Alias and Tan 2005; Çelik <i>et al.</i> 2005)
Caulobacter crescentus	Caulobacter medium, glucose	PHB	(Qi and Rehm 2001)
Escherichia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	(UHMW)PHB	(Mahishi et al. 2003; Kahar et al. 2005; Park et al. 2005a; Nikel et al. 2006; Sujatha and Shenbagarathai 2006)
Halomonas boliviensis	Starch hydolysate, maltose, maltotetraose and maltohexaose	PHB	(Quillaguaman <i>et al.</i> 2005, 2006)
Legionella pneumophila	Nutrient broth	PHB	(James et al. 1999)
Methylocystis sp.	Methane	PHB	(Wendlandt et al. 2005)
Microlunatus phosphovorus	Glucose, acetate	PHB	(Akar <i>et al.</i> 2006)
Pseudomonas aeruginosa	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	mcl-PHAs	(Hoffmann and Rehm 2004; Fernández <i>et al.</i> 2005)
Pseudomonas oleovorans	Octanoic acid	mcl-PHAs	(Durner et al. 2000; Foster et al. 2005)
Pseudomonas putida	Glucose, octanoic acid, undecenoic acid	mcl-PHAs	(Tobin and O'Connor 2005; Hartmann <i>et al.</i> 2006)
Pseudomonas putida, P. fluorescens, P. jessenii	Glucose, aromatic monomers	aromatic polymers	(Tobin and O'Connor 2005; Ward and O'Connor 2005; Ward <i>et al.</i> 2005)
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHAs	(Xu <i>et al.</i> 2005)
Rhizobium meliloti, R. viciae,	Glucose, sucrose, galactose, mannitol,	PHB	(Mercan and Beyatli 2005)
Bradyrhizobium japonicum	trehalose, xylose, raffinose, maltose, dextrose, lactose, pyruvate, sugar beet molasses, whey		
Rhodopseudomonas palustris	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV	(Mukhopadhyay <i>et al.</i> 2005)
Spirulina platensis (cyanobacterium)	Carbon dioxide	PHB	(Jau et al. 2005)
Staphylococcus epidermidis	Malt, soy waste, milk waste, vinegar waste, sesame oil	РНВ	(Wong et al. 2004, 2005)
Cupriavidus necator	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil	PHB, copolymers	(Kim et al. 1995; Kichise et al. 1999; Taguchi et al. 2003; Kahar et al. 2004; Khanna and Srivastava 2005a; Volova and Kalacheva 2005; Volova et al. 2005)
Cupriavidus necator H16	Hydrogen, carbon dioxide	PHB	(Pohlmann et al. 2006)
Cupriavidus necator H16	Hydrogen, carbon dioxide	РНВ	Volova and Kalacheva 2005; Volova <i>et al.</i> 2005) (Pohlmann <i>et al.</i> 2006)

3.3.2.1 Ralstonia eutropha

Ralstonia eutropha is a Gram-negative, facultative chemolithoautotrophic bacterium and is a model strain for the study of PHA metabolism. It is able to synthesize and accumulate P(3HB) in the cytoplasm as carbon and energy storage from simple carbon sources such as glucose, fructose and acetic acid (47, 48). In 2006, Pohlmann et al., sequenced the whole genome of R. eutropha H16 (49). Therefore, many key components of PHA metabolism in R. eutropha have been revealed (Fig 3.3). For P(3HB) biosynthesis, three key enzymes, β -ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC), are responsible for a three-step reaction starting with the condensation of two acetyl-CoA to acetoacetyl-CoA then reduces to (R)-3-hydroxybutyryl-CoA and polymerizes to P(3HB) (Pathway I in Fig 1.1). These enzymes are encoded by phaA, *phaB*, and *phaC* respectively. These genes are organized in one operon as *phaCAB* (Fig 3.4A) (50).

It was reported that *R. eutropha* accumulated large amount of P(3HB) at 76% of cell dry weight when cultured in glucose with fed-batch fermentation (51). In fact, *R. eutropha* can produce PHAs consisting of various SCL-3-hydroxyalkanoic acid (SCL-3HA), for example, P(3HB-*co*-3HP) (52) and P(3HB-*co*-3HV) by co-feeding with propionic acid (53). Moreover, Green et al., 2000 reported that *R. eutropha* can produce PHAs consisting not only SCL-3HA but also MCL-3HA (at a minor fraction) when sodium acrylate was added to the culture medium to inhibit the 3-ketoacyl-CoA thiolase activity in the final step of β -oxidation (54). The result suggested that PHA synthase of *R. eutropha* (PhaC_{*Re*}) has broad range substrate specificity and can polymerize both SCL-3HA and MCL-3HA monomers.



Figure 3.3 The elements involved in P(3HB) metabolism in *R. eutropha* **H16** [reproduced from (48) with permission from S. Karger AG].

The enzymes and proteins involved directly in P(3HB) metabolism are represented in bold text. PhaA, β -ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaZ, P(3HB) depolymerase; PhaY, 3HBoligomer hydrolase; PhaP, Phasin; PhaR, Transcriptional regulator of Phasin expression. Chitwadee Phithakrotchanakoon



Figure 3.4 The organization of *pha* genes in three native PHA-producing bacteria. (A) a complete *phaCAB* operon in *Ralstonia eutropha*; (B) two *phaCs*, *phaC1* and *phaC2*, are separated by *phaZ* in *Pseudomonas putida* KT2440 downstream of *phaJ1* and *phaJ4*; (C) *phaPCJ* operon in *Aeromonas hydrophila* ATCC 7966. *phaA*, β -ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *phaC*, PHA synthase; *phaJ*, (*R*)-specific enoyl-CoA hydratase; *phaP*, phasing (PHA granule associated protein); *phaZ*, PHA depolymerase.

3.3.2.2 Pseudomonas spp.

Some *Pseudomonas* spp. can synthesize and accumulate PHAs of various MCL-3HAs under stressed condition when grown on related-carbon sources such as plant oil, fatty acids or alkanoic acids (55-57) as well as non-related carbon sources such as glucose or gluconate (43, 58, 59). The biosynthesis routes of PHAs in Pseudomonas spp. are linked with fatty acid β -oxidation and de novo fatty acid biosynthesis pathway (pathway II and III in Fig 1.1) (60). Among many strains of Pseudomonas, an attractive strain is Pseudomonas putida KT2440. This strain is a metabolically versatile saprophytic soil bacterium which can utilize various carbon sources such as glycerol, gluconate, and fatty acids (61, 62). In 2002, the whole genome of Pseudomonas putida KT2440 was sequenced and the putative genes encoding enzymes involved in the PHA biosynthesis have been identified (63). There are two *phaC* genes (*phaC1* and *phaC2*) separated by *phaZ* (Fig 3.4B) which are similar to that from Pseudomonas strains such as P. oleovorans (64), P. aeruginosa (65), and Pseudomonas sp. strain 61-3 (66). PhaC1 of P. putida KT2440 was cloned and expressed in *E. coli* revealing its substrate specificity toward MCL-monomers: 3-hydroxyoctanoyl-CoA (C8) and 3-hydroxydecanoyl-CoA (C10) (67). Moreover, three homologs of *phaJ* (*phaJ1*, *phaJ3*, and *phaJ4*) of *P. aeruginosa* are found based on genome database search (62, 68). PhaJ1 and PhaJ4 were heterologously expressed in E. coli revealing that they exhibit high substrate specificity toward MCL-enoyl-CoA. By monitoring the gene expression level and characterization of hydratase activity in P. putida cytosolic protein, it was suggested that PhaJ4 is one of the main contributor involved in PHA biosynthesis *via* fatty acid β -oxidation (62, 69).

3.3.2.3 Aeromonas spp.

Aeromonas spp. are Gram-negative, facultative anaerobic bacteria which are found in aquatic environment (70). They are reported to be the cause of some diseases such as bacterial gastroenteritis, prostatitis, and hemolyticuremic syndromethese in invertebrates and vertebrates such as fish, frogs, bird, domestic animal, and human (11). Nonetheless, *Aeromonas strains* are intensively studied for their ability to synthesize PHA consisting of both SCL-3HA and MCL-3HA units (9, 71, 72). In fact, there are only a few bacterial strains that can produce SCL-MCL-PHAs; for example, some *Pseudomonas* strains (73, 74), *Rhodocyclus gelatinous* (75), and *Rhodococcus rubber* (76). *Aeromonas* spp. produce P(3HB-*co*-3HHx) from alkanoic acid or from plant oil. The P(3HB-*co*-3HHx) biosynthesis pathway of *Aeromonas caviae* was proposed by Fukui et al. (Fig 3.5) (13, 77). The key enzymes for P(3HB-*co*-3HHx) biosynthesis in *A. caviae* are PHA synthase (PhaC) and (*R*)-specific enoyl-COA hydratase (PhaJ). The precursors for P(3HB-*co*-3HHx) synthesis are supplied through fatty acid β -oxidation via the activity of (*R*)-specific enoyl-CoA hydratase (PhaJ) (pathway II in Fig 1.1). In 2006, the genome of *Aeromonas hydrophila* ATCC 7966 was sequenced and revealed its PHA biosynthesis genes system (70). The genes are organized in the genome as *phaPCJ* operon (Fig 3.4C) which is similar to that of *A. caviae* (77).



Figure 3.5 Proposed pathway of P(3HB-*co*-3HHx) biosynthesis in *A. caviae* from alkanoic acids or oils. (1), β -ketothiolase; (2), NADH-acetoacetyl-CoA dehydrogenase; (3), crotonase [(*S*)-specific enoyl-CoA hydratase] [reproduced from (77) with permission from American Society for Microbiology].
3.3.2.4 Recombinant Escherichia coli

Escherichia coli is a useful host strain for production of several products. There are many advantages of using *E. coli* as a producer over wild-type PHA-producing bacteria. They are: fast growth to high cell density, no special cultivated conditions required, the ability to utilize several carbon substrates, relatively easy extraction and purification of PHAs produced from *E. coli*, lack of intracellular PHA depolymerase and is safe for large scale production (78, 79). Moreover, biosynthesis of PHAs in host organisms that naturally do not produce PHAs allows modulation and regulation of the biosynthetic pathway (80). Several metabolic engineering strategies, either individually or in combination, have been used to construct recombinant *E. coli* for production of a variety of PHAs with desired compositions and properties. These include host genome manipulation, recombinant gene expression, protein engineering, external substrate manipulation and addition of inhibitor (Fig 3.6) (81).

3.3.2.4.1 SCL-PHA production

Among many type of SCL-PHAs, P(3HB) is the best characterized. Several studies have established the P(3HB) biosynthesis pathway in recombinant E. coli and has developed strategies for high production of P(3HB) (79). As mentioned above, there are two key enzymes, β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), which are involved in supplying 3-hydroxybutyryl-CoA (3HB-CoA) for PHA synthase (PhaC) (pathway I). The *phaCAB* operons of various P(3HB)-producing bacteria, for example Alcaligenes eutrophus, Methylobacterium extorquens, Rhizobium meliloti, and Streptomyces aureofaciens, have been identified and heterologously expressed in E. coli which lead to P(3HB) accumulation (28, 44-46). Moreover, the other factors that affect the biosynthesis of P(3HB) in E. coli have also been manipulated. Acetyl-CoA, serving as a precursor to P(3HB) monomer, was modulated by inactivation of the pta gene encoding a phosphotransacetylase of E. coli host genome (82). NADPH, required as a reducing power for acetoacetyl-CoA reductase (PhaB), was increased by knocking out the pgi gene encoding phosphoglucose isomersase (83) or co-transformation of *tkt* gene encoding transketolase with *phaCAB* (84).





Many approaches can be used for metabolic engineering. For example, substrate precursors can be added into the culture media. Protein evolution and mutagenesis are used to enhance the enzyme activity. Adding some inhibitors into the culture media or host cell genome manipulation can be used to inhibit some competitive pathways. Heterologous gene expression is used to introduce target enzymes into the recombinant host.

3.3.2.4.2 MCL-PHA production

MCL-PHA production is linked with fatty acid metabolism (Pathway II and III). Some intermediates of fatty acid β-oxidation and enoyl-CoA, de novo fatty acid biosynthesis including 3-ketoacyl-CoA, (S)-3-hydroxyacyl-CoA, and 3-hydroxyacl-ACP are seen to be major precursors of PHA biosynthesis. The enzymes involved are (*R*)-specific enoyl-CoA hydratase (PhaJ) (68, 77, 85, 86), 3-ketoacyl-ACP reductase (FabG) (87, 88), and 3-hydoxyacyl-ACP-CoA transferase (PhaG) (43, 89) were identified and cloned from native PHA-producing bacteria. Table 3.3 shows the list of these enzymes and other enzymes which have been successfully expressed in recombinant E. coli for PHA biosynthesis from fatty acid metabolism (41).

Metabolic engineering of fatty acid metabolism pathway for supporting PHA biosynthesis in recombinant *E. coli* is considered in two ways (individually or in combination). The former is inhibition of the enzymes in fatty acid metabolism and the later is amplification of enzymes that convert the intermediates from fatty acid metabolism pathway to monomers of PHAs. For enzyme inhibition, *fadA* and/or *fadB* mutant *E. coli* strains are popular (87, 90-93). The first successful biosynthesis of MCL-PHA in recombinant *E. coli* from fatty acids was established by expressing *phaC1* gene of *P. aeruginosa* in *fadB* mutant *E. coli* (93). In *fadB* mutant *E. coli* strain, *E. coli* MaoC which is homologous to *P. aeruginosa* (*R*)-specific enoyl-CoA hydratase (PhaJ) was found to be important for supplying (*R*)-3HA-CoA from the intermediate of β -oxidation pathway to PHA biosynthesis pathway. However, it was found that only PhaC expression in *E. coli* with a functional β -oxidation pathway did not lead to the production of PHA suggesting that the activity of *E. coli* MaoC toward enoyl-CoA is weaker than FadB in β -oxidation pathway (94).

The second way is amplification of enzymes that convert the intermediates from fatty acid metabolism pathway to monomers of PHAs. Various enoyl-CoA hydratase (PhaJ) with different substrate specificities from various natural PHA-producing bacteria have been cloned and expressed in *E. coli* (68, 77, 86, 95). Here, however, high-level expression of PhaJ is needed to compete with the β -oxidation pathway enzyme for channeling its intermediates to PHA biosynthesis pathway.

Table 3.3 List of enzymes which have been successfully expressed in recombinant E. coli for PHA biosynthesis from fatty acid metabolism [reproduced from (41) with permission from Elsevier].

Enzymes	Characteristic	Representative sources	Substrates ^a	Reference
PHA synthase				
PhaC1, PhaC2	Delementation of UA CoA.	Pseudomonads	C6-C12	[5]
PhaC	Polymenzation of HA-CoAs	Aeromonads	C4, C6	[36]
Enoyl-CoA hydratase				
PhaJ		Aeromonads	C4, C6	[21,23]
ePhaJ ^b		A. caviae	C4-C12	[25]
PhaJ1, PhaJ2, PhaJ3, PhaJ4	Hudration of apartl CoA	P. aeruginosa	C6-C12	[24,26]
MaoC	Hydrauoli of enoyi-CoA	E. coli	C6-C10	[37]
YfeX ^e		E. coli	C4-C12	[38]
PaaG ^e , PaaF ^e , YdbU ^e		E. coli	C6-C12	[39]
Ketoacyl-CoA reductase				
FabG	D 1	E. coli	C6-C12	[18-20]
RhlG	Reduction of 3-ketoacyl-CoA	P. aeruginosa	C6-C10	[18]
3-Hydroxydecanoyl-ACP:CoA tra	nsacylase			
PhaG	Commission of 2 had a second ACD to	P. putida	C6-C12	[35]
FabH, FabD	Conversion of 3-hydroxyacyi-ACP to	E. coli	C4	[40]
eFabH ^d	3-flydroxyacyi-CoA	E. coli	C4-C10	[41]

Multifunctional enzyme carrying out hydration of enoyl-CoA and dehydration of 3-HA-CoA.

^d Engineered *E. coli* FabH.

3.3.2.4.3 SCL-MCL-PHA production

Only a few bacteria with the ability to synthesize SCL-MCL-PHAs have been discovered; for example, Aeromonas caviae and Aeromonas hydrophila which produce P(3HB-co-3HHx) from alkanoic acids (9, 10) and Pseudomonas sp. 61-3 which produces random copolymer consisting of C4-C12 monomer units from gluconate (8). Metabolic pathways for production of SCL-MCL-PHAs have successfully been established in recombinant E. coli. Several combination patterns of *pha* genes have been created. For example, PHA synthase gene $(phaC_{Re})$ of R. eutropha was co-expressed with two monomer supplying enzymes encoded by β -ketothiolase (*phaA_{Re}*) and acetoacetyl-CoA reductase (*phaB_{Re}*) genes in E. coli mutant in fatty acid β -oxidation (E. coli fadB mutant LS1298). The recombinant strain produced SCL-MCL-PHAs consisting of 3HB and 3HO when using octanoate or decanoate as carbon source (14). The pathway for biosynthesis of copolymers from glucose was established in E. coli JM109 by co-expression of various mutant 3-ketoacyl-acyl carrier protein synthase III genes (fabH) with phaCl of Pseudomonas sp. 61-3 resulting in production of copolymers consisting of C4 to C10 monomers with variation of % molar fraction (15). Moreover, the hybrid pathway which combines two monomer supplying routes including PhaA and PhaB from

Bacillus sp. 256 and PhaJ from *P. aeruginosa* was constructed in *E. coli* and they were co-expressed with PhaC of *P. aeruginosa*. The recombinant strain can produce copolymers from glucose and fatty acids (96).

3.4 Biodegradation of PHAs

In the environment, PHAs can be degraded by both heat and the activity of some microorganisms such as bacteria (Bacillus sp., Pseudomonas sp., and Streptomyces sp.) and fungi (Aspergillus ustus and Penicillium sp.) (37, 97, 98). Microorganisms attack to the surface of PHA and secrete the enzyme called extracellular PHA depolymerase to solubilize PHA to its oligomers. The oligomers are then absorbed into the cell and hydrolyzed by some hydrolase to its monomers. The monomers are then used as nutrient (99). The rate of PHAs biodegradation depends on the characteristic of PHAs such as monomer compositions, crystallinity, and molecular weight as well as on the environmental conditions such as temperature, pH, and moisture. For example, P(3HB) takes a few months to be degraded in anaerobic compost but takes a year to be degraded in seawater (100-102). Besides the degradation of PHAs in the environment, PHAs used in biomedical material like surgical sutures, implant patches, and drug delivering can be degraded in the living organism by some enzymes in blood and tissue such as lipase and esterase (103, 104). The products from the PHAs degradation have no toxicity effects on the living organisms (105, 106).

3.5 Applications of PHAs

PHAs have a wide range of applications due to their properties, ranging from thermoplastic to elastomers (Fig 3.7) (107). Detailed information of three major applications including industrial, medical, and agricultural are:

3.5.1 Industrial applications

Earlier PHAs were developed for everyday-use applications. For example, P(3HB) was used to produce combs, pens, and bullets by Biomer company (Germany). Due to the gas barrier property of P(3HB-*co*-3HV), it is suitable for use in food packaging, plastic beverage bottles, coated paper and film. P(3HB-*co*-3HHx) has been used to make the flexible products such as flexible packaging, binder, and nonwovens (99).

3.5.2 Medical applications

Due to the biodegradability and biocompatibility property of PHA, the recent development has been focused on their medical applications; for example, implant materials (bone marrow scaffolds, cardiovascular patches, and surgical sutures) and drug delivery (tablets, drug controlled-release matrices) (108).

3.5.3 Agricultural applications

PHAs are useful in agriculture applications since they can be degraded in both aerobic and anaerobic soil conditions (101). PHAs have been used for coating of seeds, fertilizer, insecticide, and herbicides, for the slow release in soil. In addition they are used as mulch film and plastic containers as there is no need for removal after harvesting process (30). Fac. of Grad. Studies, Mahidol Univ.

Ph.D.(Molecular Genetics and Genetic Engineering) / 25



Figure 3.7 Applications of PHAs [reproduced from (107) with permission from Springer].

PHAs can be used to produce many kinds of plastic products such as for food packaging and fiber. Chiral-(R)-form of PHA monomers can be used as starting materials in industrial fermentation. PHAs are used as bio-implants and drug encapsulators. PHAs are catalyzed to hydroxyalkanoate methyl esters which are used as biofuels. (R)-3HB hydrolyzed from P(3HB) has therapeutic potential on Alzheimer's disease.

3.6 Glycerol

3.6.1 Glycerol from biodiesel production

Glycerol is becoming an interesting carbon source in biotechnology development since it is a by-product from biodiesel production. Recently, biodiesel which is an alternative fuel has seen an increased in production. Approximately 10% (w/w) crude glycerol was produced as a by-product from transesterification of vegetable oils and animal fat in biodiesel production (Fig 3.8). It is estimated that the world biodiesel market would come to 37 billion gallons by 2016, which means that approximately 4 billion gallons of crude glycerol will be produced. The compositions of crude glycerol are varied with biodiesel production process (types of catalyst, the nature of the starting materials, the alcohol/oil ratio, the temperature of reaction, water content, and free fatty acid content) (109). In general it is composed of glycerol as a major component, mono-/di-/tri glycerides, soap and a substantial content of methanol and salts (110). The flow chart of alkaline catalyzed biodiesel production is depicted (Fig 3.9). Although purified glycerol is used in many applications such as in food, cosmetic, and pharmaceutical industries (111), the surplus glycerol still exists, which leads to remarkable reduction in its price in the world market from 650-700 €/ton in 1996 to 150-200 €/ton in 2005 (112).



Figure 3.8 Biodiesel reaction.

Transesterification of triglycerides with methanol using NaOH as a catalyst producing biodiesel or fatty acid methyl esters (FAMEs) and glycerol.

Fac. of Grad. Studies, Mahidol Univ.



Figure 3.9 Flow chart of alkaline catalyzed biodiesel production [reproduced from (109) with permission from Springer].

Compositions of crude glycerol depend on starting materials (oil/fat, alcohol, and catalyst), the transesterification method (molar ratio of alcohol-to-oil and temperature), and the process for separation.

3.6.2 Glycerol catabolism in E. coli

Glycerol is a polyol compound that has three hydroxyl groups. *E. coli* catabolizes glycerol through the functions of glycerol transporter, ATP-dependent glycerol kinase, and glycerol-3-phosphate dehydrogenase (Fig 3.10) (113).



Figure 3.10 Glycerol catabolism in *E. coli* [reproduced from (113) with permission from American Society for Microbiology].

Glycerol catabolism in *E. coli* involves a glycerol transporter (encoded by glpF), an ATP-dependent glycerol kinase (GK encoded by glpK), glycerol-3-phosphate dehydrogenase (G3PDH encoded by glpD).

Fac. of Grad. Studies, Mahidol Univ.

3.6.3 Conversion of glycerol to PHAs

Many studies investigated the alternative uses of crude glycerol, for example, combustion for heat (114), use in animal feeding (115), and conversion by thermochemical process to higher-value products (114, 116). Moreover, glycerol can be used as a carbon feed stock for microorganism for production of value-added products such as hydrogen, ethanol, 1,3-propanediol, and polyhydroxyalkanoates (PHAs) (117, 118). Figure 3.11 shows the overview of the pathways and products converted from glycerol. Production of PHAs from glycerol has been reported using either wild-type or recombinant bacterial strains (Table 3.4)





glycerol.
from
production
PHAS
Table 3.4

Qfenstin	Teno of DU A	Onomotion mode	Culture medium	Cultivation	VHd %	AHA	Dofemeneo
menc	туре от гла	Operation more	(carbon source)	time (h)	content	(g/L)	Nelel elice
			Crude glycerol+				Kenny ST et al
P. putida GO16	MCL-PHA	Fed-batch	Sodium	48	I	5.7	
			terephthalate				(611)(7107)
Mixed microbial					62		Moralejo-Garate H
commuties	(dnc)1	red-Datch	CIUNE BUYCEIOL	I	0 /	I	et al., (2011) (120)
Recombinant E. coli			Pure glycerol+			• •	
(deletion: <i>ptsG</i> , <i>sdhA</i> ,		Flask (50 mL)	decanoate	I	4.5	0.2	Kang Z et al. (2011)
and <i>pta</i> genes,	MCL-PHA	Ead batab	D				(121)
heterologous expression:			rure gryceror ⊤	> 90	5.6	0.5	
phaCl ofP. aeruginosa		(7 5)	decalloate				
		-	Pure glycerol		40.5-44.8	1.9-2.3	Kawata Y and Aiba
Hatomonas sp. K.M-1	P(3HB)	Flask (20 mL)	Crude glycerol	00	39	1.6	S(2010)(122)

Ctracity	A DU A	Onomition mode	Culture medium	Cultivation	% PHA	PHA	Dofennes
IIIBUC	туре от гла	орегацоп шоце	(carbon source)	time (h)	content	(g/L)	Veletence
Bacillus sonorensis		Ē			71.8		v
Halomonas	P(3HB)	FlasK (100 mJ)	Crude glycerol	96	75		Shirvastav A et al (2010)/(123)
hydrothermalis					C	1	CC 41.) (7 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
		Flask			010	9	
Burkholderia cepacia		(100 mL)	- - 7	90	6.18	۵.4 ۲	Zhu C et al., (2010)
ATCC 17759 (pathogen)	P(3HB)	Fed-batch	Unde grycerol	<		t	(124)
		(200 L)		120	51.4	4./	
		Fed-batch	Pure glycerol		12	1.4	Andreesen B
Recombinant E. coli	P(3HP)	(2 L)	Crude glycerol	92	5	0.3	et al., (2010) (125)
				48	53.6	1.7	г шу ү; тш
J		ШТ	rure gryceror	96	80.1	2.8	IDIALIIII MH AIG
200eueua aenirijican	(dnc)4		Pure glycerol +	48	79.7	2.9	Stellibucher A, 2010
			NaCl	96	87	4.2	(071)

Т

Т

Table 3.4 PHAs production from glycerol (Cont.).

Т

-

Character of the second se	T _{erro} of DITA	Orona tion mode	Culture medium	Cultivation	% PHA	PHA	Defenses
IIIEINC	туре от гна	Operation mode	(carbon source)	time (h)	content	(g/L)	Kelerence
			Pure glycerol +				
Burkholderia sp. USM			palm oil	ĉ			Chee JY et at., 2010
(JMC 15050)	(quc)1	I	derivertivies +	7/	/7-77		(3)
			fatty acid				
			Pure glycerol	34	62	51.2	Cavalheiro et al.,
Cupriaviaus necator	P(3HB)	red-batch	Crude glycerol		50	38.1	2009 (2)
							Ibrahim MH and
соренена аети-прсан <i>s</i>	P(3HB)	Fed-batch	Pure glycerol	50	67	54.3	Steinbuchel A, 2009
1 M M							(127)
		Batch 5.6 L-		or	ç	ч С	
		reactor	-	40	47	с.c	Pablo I et al., 2008
Kecombinant <i>E. con</i>	P(3HB)	Fed-batch 5.6 L-	Pure grycerol	U9	15	10.0	(128)
		reactor		00	10	10.0	

Table 3.4 PHAs production from glycerol (Cont.).

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation	% PHA content	PHA	Reference
Recombinant E. coli		flask			9.8	0.6	
(harboring <i>phaBAC</i> of							
<i>Azotobacter</i> sp strain		5.6 L-bioreactor			46.1	3.4	
FA8)			-	ç			Almeida A et al.,
Recombinant E. coli	P(3HB)	Flask	Pure glycerol	84	38.2	3.1	2007(129)
(harboring <i>phaBACP</i> of				•			
<i>Azotobacter</i> sp strain		5.6 L-bioreactor			51.9	7.9	
FA8)							
Paracoccus denitrificans			Pure glycerol		70		Mothes G et al.,
and Cupriavidus necator	P(3HB)	Fed-batch	Crude glycerol	ı	48	ı	2007(130)
High osmophilic	P(3HB- <i>co</i> -	Batch 42-L	Crude glycerol +	01	שע	с у <u>г</u>	Koller et al., 2005
microorganism	3HV)	bioreactor	peptone	1 / 0	0/	10.2	(131)
Pseudomonas NRRLB-	(dire/d	Flask		Ċ			Ashby RD et al.,
14682	r(dhc)r	(500 mL)	CTude gryceror	7/	13-2/	ı	2004(132)

Table 3.4 PHAs production from glycerol (Cont.).

t.).	
cerol (Con	
ion from gly	
As product	
ble 3.4 PH	
Ta	

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
Recombinant E. coli	P(3HB)	Flask (50 mL)	Pure glycerol + peptone	48	60	I	Mahishi LH et al., 2003 (133)
Recombinant Salmonella enteric	P(3HB- <i>co</i> - 3HV)	Flask	Pure glycerol	,	27	I	Aldor IS et al., 2002 (134)
Methylobacterium rhodesianum	P(3HB)	Flask	Pure glycerol + casein peptone	92	39	·	Borman EJ and Roth M, 1999 (135)

CHAPTER IV MATERIALS

4.1 Bacterial strains

4.1.1 PHA-producing strains

Ralstonia eutropha (TISTR 1095), a PHA-producing bacteria, was obtained from the Thailand Institute of Scientific and Technological Research, Thailand. Two other strains of PHA-producing bacteria which are *Aeromonas hydrophila* (ATCC 7966), and *Pseudomonas putida* (ATCC 47054), were obtained from the American Type Culture Collection, USA.

4.1.2 Escherichia coli strains

Escherichia coli DH5 α [*sup*E 44, Δ *lac* U169, (Φ 80 *lac*Z Δ M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1], was used as a host for plasmid propagation.

Escherichia coli BL21(DE3) [F⁻ ompT gal dcm lon hsdS_B($r_B^- m_B^-$) λ (DE3 [lacI lacUV5-T7 gene 1 *ind*1 *sam*7 *nin*5])], was used as a host for protein expression.

4.2 Plasmid vectors

Plasmid vectors used in this study are shown in Figures 4.1-4.3. The plasmid pTZ57R/T vector (Fermentas, Lithuania) is used for all cloning steps (Fig 4.1). Two vectors, pETDuet-1 and pCDFDuet-1 (Novagen, Germany) were employed for PhaA, PhaB, PhaC and PhaJ expression (Fig 4.2 and 4.3) and were kindly provided by Dr. Sarin Chimnaronk.

Chitwadee Phithakrotchanakoon



Figure 4.1 A physical map of pTZ57R/T vector (taken from Fermentas website: http://www.fermentas.com/en/products/all/molecular-cloning/kits/k121-instaclone-pcr-cloning?print).

The figure illustrates a TA cloning vector, pTZ57R/T, containing *lacZ* gene, multiple cloning sites, origin of replication in *E. coli* and ampicillin resistant gene.





Figure 4.2 A physical map of pETDuet-1 vector (taken from Novagen's user protocol TB340 Rev. E 0305).

The figure illustrates a pETDuet-1 plasmid containing 2 multiple cloning sites driving by separated T7 promoters, His_6 -tag, T7 transcription terminator, ampicillin resistant gene and ColE1 (pBR322) origin of replication.

Chitwadee Phithakrotchanakoon





Figure 4.3 A physical map of pCDFDuet-1 vector (taken from Novagen's user protocol TB340 Rev. E 0305).

The figure illustrates a pCDFDuet-1 plasmid containing 2 multiple cloning sites driving by separated T7 promoters, His₆-tag, T7 transcription terminator, streptomycin resistant gene and CloDF13 origin of replication.

4.3 Synthetic oligonucleotides

All nucleotide primers were synthesized by BioDesign Co., Ltd., Thailand.

4.4 Culture media

4.4.1 Culture media for native PHA-producing bacteria

Ralstonia eutropha and *Aeromonas hydrophila* were cultivated in nutrient broth medium (BD Difco, USA) at 25°C and 30°C, respectively, with continuous shaking at 200 rpm. *Pseudomonas putida* was cultivated in LB medium at 37°C with continuous shaking at 200 rpm.

4.4.2 Culture media for *E. coli* harboring pTZ57R/T vector (for cloning)

E. coli DH5 α , was grown in Luria Bertani (LB) medium [1% (w/v) tryptone (Difco, USA), 1% (w/v) NaCl (Carlo Erba, France), and 0.5% (w/v) yeast extract (Difco, USA). For LB plate agar, 1.5% (w/v) bacteriology agar was added. The *E. coli* transformants were grown in LB containing 100 µg/mL of ampicillin. For blue/white colonies screening, 25 µl of a stock solution of 20 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) in dimethylformamide and 50 µl of a stock solution of 100 mg/mL isopropylthio- β -D-galactoside (IPTG) (Thermo Scientific, USA) were spreaded on the plate agar.

4.4.3 Culture media for *E. coli* harboring pETDuet-1 and/or pCDFDuet-1 vector (for expression)

E. coli BL21(DE3), was cultivated in Luria Bertani (LB) medium containing 100 μ g/mL ampicillin and/or 50 μ g/mL streptomycin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous protein expression.

4.4.4 Culture media for P(3HB) biosynthesis

Recombinant *E. coli* harboring *phaA*, *phaB* and *phaC* were cultivated in M9 medium [0.3% (w/v) KH₂PO₄ (Carlo Erba, France), 0.05% (w/v) NaCl (Carlo Erba, France), 0.1% (w/v) NH₄Cl (Carlo Erba, France), 0.68% (w/v) Na₂HPO₄ (Carlo Erba, France), 1 mM MgSO₄ (Carlo Erba, France), 1 mM CaCl₂ (Carlo Erba, France)] with or without 0.1% (w/v) yeast extract (Difco, USA). Pure glycerol or crude glycerol or glucose was used as sole carbon source with 100 μ g/mL ampicillin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous protein expression.

4.4.5 Culture media for SCL-MCL-PHAs biosynthesis

Recombinant *E. coli* harboring *phaA*, *phaB*, *phaC* and *phaJ* were cultivated in M9 medium containing pure glycerol or crude glycerol and sodium dodecanoate (Sigma, USA) whose concentration ratio was varied as indicated in the method section, 50 μ g/mL ampicillin and 50 μ g/mL streptomycin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous proteins expression. All antibiotics were purchased from Sigma, USA.

4.5 Chemicals

Crude glycerol was kindly provided by Trang Palm Oil Co. Ltd., Trang, Thailand. Other analytical grade chemicals and solvents were purchased from Carlo Erba, (France), Fluka, (Switzerland), Sigma, (USA), Merck, (Germany) or Thermo Scientific, (USA).

4.6 Enzymes

Restriction enzymes and other modification enzymes were purchased from Fermentas (Lithuania).

4.7 Antibodies

Monoclonal Anti-polyHistidine, clone HIS-1, produced in mouse and Anti-Mouse IgG (Whole molecules)-Alkaline Phosphatase, produced in goat were purchased from Sigma (USA).

4.8 Commercial kits

GeneJET[™] Plasmid Miniprep kit (Fermentas, Lithuania), QIAquick PCR Purification kit (QIAGEN, German).

CHAPTER V METHODS

5.1 Genomic DNA extraction

The genomic DNA was isolated from R. eutropha (TISTR 1095), A. hydrophila (ATCC 7966) and P. putida KT2440 (ATCC 47054). Bacterial colonies were resuspended in 360 µL TE buffer (100 mM Tris-HCl pH 8.0, 30 mM EDTA pH 8.0). Then, 20 μ L of lysozyme (100 mg/mL) was added and incubated at 37°C for 2 h. Next, 40 µL of 10% SDS was added and incubated at 55°C for 30 min. One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added then mixed and centrifuged at 16,400 xg for 15 min at room temperature. The top phase was transferred to a new 1.5 mL tube and 1 volume of chloroform: isoamyl alcohol (24: 1) was added. After centrifugation at 16,400 xg for 15 min at room temperature, the top phase was transferred to a new 1.5 mL tube and 0.1 volume of 3 M sodium acetate was added and mixed gently. The genomic DNA was precipitated by mixing with 2 volumes of cold ethanol and kept at -20°C for 30 min. The precipitated genomic DNA was collected by centrifugation at 16,400 xg for 30 min at 4°C. Then, the DNA pellet was washed twice with 70% cold ethanol and centrifuged at 16,400 xg for 5 min at 4° C. The DNA pellet was air dried and dissolved in 30 µL distilled water. The extracted genomic DNA solution was stored at -20°C.

Spectrophotometry was used to determine the quality and concentration of DNA. The concentration of DNA was analyzed by measuring the absorbance at 260 nm (A_{260}). The purity of DNA was determined by the ratio of A_{260}/A_{280} . It should be around 1.8.

Agarose gel electrophoresis was also used to analyze the quality and concentration of DNA. Agarose gel was prepared by melting 0.8% (w/v) of agarose in 1X TAE buffer (Tris-acetate EDTA buffer; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The melted gel was poured into a tray and allowed to set at room temperature. For electrophoresis, the DNA solution was mixed with loading dye (25% glycerol,

60 mM EDTA and 0.25% bromophenol blue) then loaded into the well of set gel and ran at a constant voltage at 100 volts in 1X TAE buffer. After running, the gel was stained by immersing in 2.5 μ g/mL of ethidium bromide solution for 5 min then destained in water for 10 min. The migration of the genomic DNA was visualized on top of an ultraviolet (UV) transilluminator.

5.2 Competent cell preparation

In this study, *E. coli* strains DH5 α and BL21(DE3) were used. The competent cells were prepared as described below. One colony of *E. coli* was inoculated into 5 mL of LB medium and grown at 37°C with shaking at 200 rpm for 12 h. Then 1 mL of the cultured cells was inoculated into 50 mL of fresh LB medium and grown at 37°C with shaking at 200 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.45. Then cultured cells were transferred into a sterile 50 mL polypropylene centrifuge tube and chilled on ice for 10 min. The cells were collected by centrifugation at 2,500 x*g* for 10 min at 4°C. Then the supernatant was discarded. The pellet was resuspended in 10 mL of ice-cold 0.1 M CaCl₂ and chilled on ice for 10 min. The cells were again collected by centrifugation at 2,500 x*g* for 10 min at 4°C and resuspended in 2 mL of ice-cold 0.1 M CaCl₂. Sterile glycerol was added to final concentration 30% (v/v). After chilled on ice for 10 min, the competent cells were aliquot into 1.5 mL tube, snaped-frozen in liquid nitrogen and kept at -80°C until used.

5.3 Construction of recombinant plasmids

5.3.1 Construction of pETDuet-ABCs

Oligonucleotide primers were designed based on genome sequence data of *R. eutropha*, *A. hydrophila*, and *P. putida* KT2440. The gene IDs of $phaA_{Re}$, $phaB_{Re}$, $phaC_{Re}$, $phaC_{Ah}$, $phaC_{2Pp}$, $phaJ1_{Pp}$, $phaJ4_{Pp}$, and $phaJ_{Ah}$ are 4249783, 4249784, 4250156, 4488480, 1041897, 1046178, 1045908, and 4488481, respectively.

All cloning steps are described as in Figure 5.1. β -ketothiolase gene (*phaA*) and NADPH-dependent acetoacetyl-CoA reductase (*phaB*) were designed to

cloned into MCS1 of pETDuet-1 expression vector (Fig 5.2A). phaA was designed to fused with His₆-tag of pETDuet-1 vector at N-terminus whereas phaB was designed to fused with His₆-tag at C-terminus. Specifically, phaA and phaB were PCR amplified together as one fragment from the genomic DNA of R. eutropha. The oligonucleotide primers including AB(F)/EcoRI and AB(R)/AfIII were used (Table 5.1A). The amount of genomic DNA and the PCR condition are as described in Tables 5.2 and 5.3, respectively. Amplified product was analyzed on agarose gel electrophoresis and DNA band corresponding to the size of target product was purified from agarose gel using QIAquick PCR Purification kit (QIAGEN, Germany). Next, the amplified gene fragment was ligated into pTZ57R/T cloning vector to create pTZ57R/T- AB_{Re} . Then the ligation reaction was transformed into E. coli DH5a via heat-shock method. Blue/white colony screening was used to determine the candidate transformants. Next, the plasmids were isolated from several transformants and subjected to sequencing. Then, PCR amplification was performed by using $pTZ57R/T-AB_{Re}$ as template and AB(F)/EcoRI and ABH6(R)/AfIII as primers (Table 5.1B). The amplified product was analyzed on agarose gel electrophoresis. The expected amplicon was purified from agarose gel and digested with EcoRI and AflII restriction enzymes then purified by QIAquick PCR Purification kit (QIAGEN, German). Next, the amplified gene fragment was ligated into MCS1 of pETDuet-1 expression vector to create pETDuet- AB_{Re} . Then the ligation was transformed into E. coli DH5a. After the gene sequences were confirmed, the recombinant plasmids were used to transform into the expression hosts E. coli BL21(DE3).

Three different origins of PHA synthase genes (*phaCs*) were used in this study. They are: *phaC* of *R. eutropha* (C_{Re}), *phaC2* of *P. putida* (C_{2pp}) and *phaC* of *A. hydrophila* (C_{Ah}). Each of them was designed to fuse with His₆-tag at N-terminus and cloned into MCS2 of pETDuet-1 expression vector. Specifically, each *phaC* was PCR amplified from the genomic DNA of the corresponding strains to generate the gene fragments of *phaC_{Re}*, *phaC2_{Pp}*, and *phaC_{Ah}*-H6. The oligonucleotide primers, the amount of genomic DNA and the PCR conditions are as described in Tables 5.1A, 5.2 and 5.3, respectively. The amplified product was analyzed on agarose gel electrophoresis and the band corresponding to the size of target product was purified from agarose gel using QIAquick PCR Purification kit (QIAGEN, German). Next, the

amplified gene fragments were ligated into pTZ57R/T cloning vector to create pTZ57R/T- C_{Re} , pTZ57R/T- C_{2PD} , and pTZ57R/T- C_{Ah} -H6, respectively. Then the ligation reactions were transformed into E. coli DH5a. Blue/white colony screening was used to determine the candidate transformant clones. Next, the plasmids were isolated from several transformants and subjected to sequencing. Then, the PCR amplification was performed by using pTZ57R/T- C_{Re} and pTZ57R/T- C_{2Pp} as template. Oligonucleotide primers used in the PCR are shown in Table 5.1B. The amplified product was analyzed by agarose gel electrophoresis. Next, the amplified products, C_{Re} -H6 and C_{2Pp} -H6, were digested with MunI/AvrII and BglII/AvrII restriction enzymes, respectively. Then the fragments were ligated into MCS2 of pETDuet- AB_{Re} to create pETDuet- $AB_{Re}C_{Re}$ and pETDuet- $AB_{Re}C_{2p}$, respectively. Then, to create pETDuet- $AB_{Re}C_{Ah}$, pTZ57R/T- C_{Ah} -H6 was digested with MunI and C_{Ah} -H6 fragment was ligated into MCS2 of pETDuet- AB_{Re} . Next, the AvrII and the ligation reaction was transformed into E. coli DH5a. After the gene sequences were confirmed, the recombinant plasmids were used to transform into the expression hosts *E. coli* BL21(DE3).

5.3.2 Construction of pCDFDuet-Js

Three (*R*)-specific enoyl-CoA hydratase genes (*phaJs*) from three different microorganisms were used in this study. They are: *phaJ1* of *P. putida* ($J1_{Pp}$), *phaJ4* of *P. putida* ($J4_{Pp}$) and *phaJ* of *A. hydrophila* (J_{Ah}). Each of them was designed to fuse with His₆-tag at the N-terminus and then cloned into MCS1 of pCDFDuet-1 expression vector (Fig 5.2B). The cloning steps were similar to the construction of pETDuet- $AB_{Re}C_{Ah}$. Specifically, *phaJ1_{Pp}* was cloned *via* the *Bam*HI and *Hind*III sites, while *phaJ4_{Pp}* and *phaJ_{Ah}* were cloned *via* the *Eco*RI and *Hind*III sites. Therefore, three recombinant plasmids were created: pCDFDuet- $J1_{Pp}$, pCDFDuet- $J4_{Pp}$, and pCDFDuet- $J4_{Pp}$.

Genomic DNA of A. hydrophila	$\downarrow pha C_{Ah}-H6 \text{ amplification} \\ \downarrow \text{ from genomic DNA}$	Ligation in pTZ57R/T	$pTZ57R/T-C_{Ah}-H6$	Transformation into \overleftarrow{E} coli DH5 α	Plasmid screening	Digestion with $\bigvee MunI$ and $AvrII$	$R_{R_{e}} \mid \text{Ligation in MCS2 of pETDuet-}AB_{R_{e}} \land \text{tat }Mun1 \text{ and }AvrII.$	pETDuet- $AB_{Re}C_{Ah}$	Transformation into $\downarrow E. coli DH5\alpha$	Plasmid isolation ↓ and sequencing
Genomic DNA of P. putida	$\checkmark phaC2_{Pp}$ amplification from genomic DNA	Ligation in pTZ57R/T	pTZ57R/T-C2 _{Pp}	Transformation into $\checkmark E. \ coli \ DH5\alpha$	Plasmid screening	$\bigvee phaC2_{Pp}$ -H6 amplification $\bigvee \text{from pTZR/T-}C_{Re}$	$B_{Re} \mid \text{Ligation in MCS2 of pETDuet-}AB$ $\forall \text{ at } B_g/\Pi \text{ and } A\nu H$.	$pETDuet-AB_{Re}C2_{Pp}$	Transformation into $\checkmark E. \ coli$ DH5 α	Plasmid isolation
Genomic DNA of R. eutropha	$\downarrow phaC_{Re} \text{ amplification} \\ \uparrow \text{ from genomic DNA}$	Ligation in pTZ57R/T	$pTZ57R/T-C_{Re}$	Transformation into $E. \ coli \ DH5\alpha$	Plasmid screening ↓ and sequencing	$\downarrow phaC_{Re}$ -H6 amplification \downarrow from pTZR/T- C_{Re}	Ligation in MCS2 of pETDuet- A at <i>Mun</i> I and <i>Avr</i> II.	pETDuet- $AB_{Re}C_{Re}$	Transformation into $E. \ coli \ DH5\alpha$	Plasmid isolation
Genomic DNA of R. eutropha	$\downarrow phaAB_{Re}$ amplification \downarrow from genomic DNA	Ligation in pTZ57R/T	pTZ57R/T-AB _{Re}	$\checkmark E. coli DH5\alpha$	Plasmid screening	$\downarrow phaAB_{Re}$ -H6 amplification \downarrow from pTZR/T-AB_{Re}	Ligation in MCS1 of pETDuet-1 \checkmark at <i>Eco</i> RI and <i>AfII</i> .	pETDuet- AB_{Re}	Transformation into $\checkmark E. coli DH5 \alpha$	Plasmid isolation

Figure 5.1 A schematic representation of overall procedure for construction of pETDuet-ABCs.

Fac. of Grad. Studies, Mahidol Univ.

Ph.D.(Molecular Genetics and Genetic Engineering) / 47



Figure 5.2 Physical map of recombinant plasmids constructed in this study. (A) pETDuet-*ABC*s and (B) pCDFDuet-*J*s. *Re, R. eutropha*; *Ah, A. hydrophila*; *Pp, P. putida* KT2440.

Length Tm **Target DNA** Primer name Sequence (nt) (°C) 5' TCTAGAATTCGACTGACGTTGTCATCGTATCCG 3' AB(F)/EcoRI 33 63.2 phaA and phaB **Eco**RI of R. eutropha 5' CGA<u>CCTTAAG</u>TCAGCCCATATGCAGGCC 3' (AB_{Re}) AB(R)/AflII 28 64.3 AflII 5' TCGACAATTGGCATCACCATCATCACCACAGCCAA PhaC of C_AH6(F)/MunI 69.9 MunI His₆-tag 46 A. hydrophila TCATCTTACGG 3' with His6-tag 5' AGTT<u>CCTAGG</u>TCATGCTGGCTCCTCCTC 3' $(C_{Ah}-H_6)$ C_A(R)/XmaJI 28 64.3 XmaJI phaC of $C_R(F)$ 5' ATGGCGACCGGCAAAGGCGC 3' 20 60.0 R. eutropha 5' TCATGCCTTGGCTTTGACGTATCGCC 3' 61.1 $C_R(R)$ 26 (C_{Re}) phaC2 of 5' ATGACAGACAAACCGGCCAAAGGATCG 3' $C2_P(F)$ 27 61.3 P. putida 5' TCATCGGGTCAGCACGTAGGTGCCTG 3' $C2_P(R)$ 26 64.3 $(C2_{Pp})$ 5' <u>GGATCC</u>GTCCCAGGTCACCAACACGCCTTAC 3' J1_P(F)/BamHI 31 68.3 PhaJ1 of **BamHI** P. putida 5' AAGCTTTCAGCTCGCCACAAAGTTCG 3' (JI_{Pp}) J1_P(R)/HindIII 59.5 26 HindIII 5' TCTA<u>GAATTC</u>GCCCCATGTACCGGTTACAGAGC 3' J4_P(F)/EcoRI 33 65.6 PhaJ4 of **EcoRI** P. putida 5' AGTC<u>AAGCTT</u>TCAGACAAAACAGAGCGACAG 3' $(J4_P p)$ $J4_P(R)/HindIII$ 61.7 31 HindIII 5' TCTAGAATTCGAGCATGCCTCCCTTCGAGGTG 3' J_A(F)/EcoRI 32 65.7 PhaJ of **EcoRI** A. hydrophila 5'AGTCAAGCTTTCAGCCGAACTTCACCACGGCTTC 3' (J_{Ah}) J_A(R)/HindIII 34 66.8 HindIII

Table 5.1 (A) Oligonucleotide primers.

Tangat DNA	Duimon nome	Sacurance	Length	Tm
Target DNA	r miner name	Sequence	(nt)	(°C)
phaA and phaB of R. eutropha	AB(F)/EcoRI	5' TCTA <u>GAATTC</u> GACTGACGTTGTCATCGTATCCG 3' <i>Eco</i> RI	33	63.2
with His ₆ -tag $(AB_{Re}$ -H ₆)	ABH6(R)/AflII	5' GTAC <u>CTTAAG</u> TCA <u>ATGATGGTGGTGATGGTG</u> GC <i>Afl</i> II His ₆ -tag CCATATGCAGGCCGCCGTT 3'	52	74.0
phaC of R. eutropha	C _R H6(F)/MunI	5' TCGA <u>CAATTG</u> G <u>CATCACCATCATCACCAC</u> GCGA <i>Mun</i> I His ₆ -tag CCGGCAAAGGCGC 3'	46	74.3
$(C_{Re}-H_6)$	C _R (R)/XmaJI	5'AGTT <u>CCTAGG</u> TCATGCCTTGGCTTTGACGTATC 3' XmaJI	33	64.4
phaC2 of P.putida with Hise-tag	C2 _P H6(F)/ <i>Bgl</i> II	5' ACGT <u>AGATCTCCATCACCATCACCAC</u> ACAG Bg/II His ₆ -tag ACAAACCGGCCAAAGG 3'	49	72.1
$(C2_{Pp}-H_6)$	C2 _P (R)/XmaJI	5' GTAC <u>CCTAGG</u> TCATCGGGTCAGCACGTAGG 3' <i>Xma</i> JI	30	67.1

Table 5.1 (B) Oligonucleotide primers (Cont.).

	Table	5.2	Genomic	DNA	template fo	or target P	PCR fragmen
--	-------	-----	---------	-----	-------------	--------------------	--------------------

PCR fragment	Template	
i ek tragment	Name	Amount (ng)
<i>phaA</i> and <i>phaB</i> of <i>R</i> . <i>eutropha</i> (AB_{Re})	Genomic DNA of <i>R. eutropha</i>	1.5
phaC of A. hydrophila with His6-tag (CAh-H6)	Genomic DNA of A. hydrophila	400
phaC of R. eutropha (C_{Re})	Genomic DNA of R. eutropha	1.5
$phaC2$ of P . $putida(C2_{Pp})$	Genomic DNA of P. putida	1.5
$phaJ1$ of P . $putida (J1_{Pp})$	Genomic DNA of P. putida	200
$phaJ4$ of P . $putida$ $(J4_{Pp})$	Genomic DNA of P. putida	200
$phaJ$ of A. hydrophila (J_{Ah})	Genomic DNA of A. hydrophila	90

					štep				
PCR fragment	Pre-denat	turation		Amplif	ication		Final ext	tention	Polymerase
		Number	Denaturation	Annealing	Extension	Number		Number	enzyme
		of cycle	(°C/min)	(°C/min)	(^o C/min)	of cycle		of cycle	
<i>phaA</i> and <i>phaB</i> of <i>R. eutropha</i> (<i>AB_{Re}</i>)	94 (1)	1	94 (1)	50 (1)	72 (1:45)	30	72 (10)	1	DyNAzyme
<i>phaA</i> and <i>phaB</i> of <i>R. eutropha</i> with His_{6} -tag $(AB_{R_{e}}-H_{6})$	'	1	98 (0:10)	9	8 (2)	30	1		PrimeSTAR HS
PhaC of A. hydrophila with His ₆ -tag (C _{Ah} -H ₆)	95 (3)	1	95 (1)	55 (1)	72 (3:30)	30	72 (5)	1	Pfu
phaC of R. eutropha (C_{Re})	94 (2)	1	94 (1)	54 (1)	72 (1:45)	30	71 (10)	1	DyNAzyme
phaC of R. eutropha with His_{6} -tag (C_{Re} - H_{6})	94 (2)	1	94 (1)	50 (1)	72 (1:50)	30	72 (10)	1	DyNAzyme
phaC2 of P. putida (C2 _{Pp})	94 (2)	1	94 (1)	65 (1)	72 (1:45)	30	72 (10)	1	DyNAzyme
<i>phaC2</i> of <i>P. putida</i> with His ₆ -tag (<i>C</i> 2 _{<i>Pp</i>} -H ₆)	94 (2)	1	94 (1)	50 (1)	72 (1:50)	30	72 (10)	1	DyNAzyme
<i>PhaJ1</i> of <i>P</i> . <i>putida</i> (JI_{Pp})	95 (5)	1	95 (1)	60 (0:30)	72 (0:20)	30	72 (10)	1	DyNAzyme
PhaJ4 of P. putida (J4 _P p)	95 (3)	1	95 (1)	50 (0:30)	72 (0:30)	30	72 (10)	1	DyNAzyme
PhaJ of A. hydrophila (J_{Ah})	95 (3)	1	95 (1)	53 (1)	72 (0:30)	30	72 (5)	1	DyNAzyme

Table 5.3 PCR condition used for amplifying target DNA fragment.

5.4 Recombinant protein expression

E. coli BL21(DE3) was used as a host for protein expression and PHA production. The recombinant plasmids, pETDuet- $AB_{Re}C_{Re}$, pETDuet- $AB_{Re}C_{Ah}$, and pETDuet- $AB_{Re}C_{2pp}$, were transformed into *E. coli* BL21(DE3) generating *E. coli*-AB_{Re}C_{Re}, *E. coli*-AB_{Re}C_{Ah}, and *E. coli*-AB_{Re}C_{2pp}, respectively. The recombinant plasmids, pCDFDuet- JI_{Pp} , pCDFDuet- $J4_{Pp}$, and pCDFDuet- J_{Ah} were transformed into *E. coli* BL21(DE3) generating a recombinant strains namely *E. coli*-J1_{Pp}, *E. coli*-J4_{Pp}, and *E. coli*-J_{Ah}. Moreover, the recombinant plasmids: pETDuet-*ABC*s and pCDFDuet-*Js* were co-transformed into *E. coli* BL21(DE3) generating nine recombinant strains namely *E. coli*-ABC_{Re}J1_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Re}J4_{Pp}, *E. coli*-ABC_{Ah}J1_{Pp}, *E. coli*-ABC_{Ah}J4_{Pp}, *E. col*

To investigate the expression ability of recombinant proteins in *E. coli*, several transformant clones of each strain were cultivated in LB supplemented with appropriate antibiotics (100 µg/mL of ampicillin for *E. coli*-ABCs, and 50 µg/mL of streptomycin for *E. coli*-Js, and 50 µg/mL of ampicillin and 50 µg/mL streptomycin for *E. coli*-ABCsJs), overnight (16 h) at 37°C, 200 rpm. The overnight cultures were inoculated into fresh LB containing appropriate antibiotics (5% inoculums) and cultured at 37°C with shaking 200 rpm until OD₆₀₀ reached 0.6. Isopropyl-thio- β -D-galactopyranoside (IPTG) was added to the cultures as an inducer with final concentration of 1 mM. The cell cultures were grown at the same conditions for 3 h. Then they were harvested by centrifugation. Cell pellets were directly subjected to analyze by SDS-PAGE or stored at -20°C until used.

5.5 Protein analysis

5.5.1 SDS-PAGE

Protein expression profile was analyzed by SDS-PAGE. The harvested cells were mixed with 5x loading buffer [10% (w/v) SDS, 10 mM β -mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris-HCl, pH 6.8, and 0.05% (w/v) bromophenol blue). The reaction was boiled for 10 min, then vortexed. Next, it was centrifuged to obtain clear

supernatant and loaded into 12% SDS-PAGE. The ingredients for SDS-PAGE preparation is shown in Table 5.4. The protein profile was resolved in 1x Tris-glycine running buffer [25 mM Tris-HCl, 250 mM glycine and 0.1% (w/v) SDS] at constant current of 15 mA per gel. After electrophoresis, the gel was stained in staining buffer [0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, and 10% (v/v) acetic acid] for 30 min with mild shaking. Then, the gel was destained in destaining solution [10% (v/v) methanol and 10% (v/v) acetic acid] with shaking until excess dye was removed.

Preparation of 5% of stacking SDS-PAGE		
Solution	Volume (µl)	
30% Acrylamide ^a	333	
H ₂ O	1,500	
1 M Tris-HCl pH 6.8	625	
10% SDS	25	
10% APS	15	
TEMED	3	
Total volume	2,501	
Preparation of 12% of separating SDS-PAGE		
Solution	Volume (µl)	
30% Acrylamide ^a	2,400	
H ₂ O	2,010	
1 M Tris-HCl pH 8.8	1,500	
10% SDS	60	
10% APS	30	
TEMED	3	
Total volume	6,003	

Table 5.4 Components of SDS-PAGE for protein determination.

^aAcrylamide, N,N'-methylene-bis-acrylamide 29: 1 (w/w); APS, ammonium persulphate; TEMED, N,N,N',N'-tetramethyl-ethylenediamine

5.5.2 Western blot analysis

Western blot analysis was performed to verify the specificity of recombinant proteins. After the proteins were electrophoresed into 12% SDS-PAGE, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane by wet-blotting. The electroblotting was performed at constant voltage of 100 volts for 90 min in 1x transfer buffer [25 mM Tris-base, 190 mM glycine, 20% (v/v) methanol, and 0.05% (v/v) SDS]. Non-specific binding was blocked with 5% skim milk in PBS, pH 7.4 (8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of Na₂HPO₄, and 0.24 g/L of KH₂PO₄) for 2 h then washed by PBST [PBS with 0.1% (v/v) Tween-20] for 10 min. Next the membrane was probed with monoclonal antipolyHistidine in 5% skim milk in PBS (1:3,000 dilutions) for 1 h. The membrane was washed 3 times with PBST for 10 min each then probed with secondary antibody, anti-Mouse IgG (whole molecules)-alkaline phosphatase in 5% skim milk in PBS (1:30,000 dilution) for 2 h. Then, the membrane was washed once with PBST for 10 min and twice more with PBS for 10 min each. The membrane was incubated in the colorimetric detection solutions, 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium chloride (BCIP/NBT) substrate, for 30 min.

5.6 Crude glycerol characterization

Crude glycerol used in this study was obtained from a local biodiesel production plant (Trang Palm Oil Co. Ltd., Trang, Thailand) which was the by-product of alkaline (NaOH)-catalyzed transesterification of palm oil with methanol. Methanol in the crude glycerol was quantified using a gas chromatograph GC-6850 equipped with a flame ionization detector and a HP-Innowax column (30 m x 0.32 mm I.D. x 0.25 μ m film thickness) (Hewlett Packard, Santa Clara, CA). Glycerol and mono-/di-/tri-glycerides were measured using a gas chromatograph GC-2010 (d) equipped with a flame ionization detector and a type DB-1HT column (10 m x 0.32 mm I.D. x 0.1 micron film thickness) (Agilent, Santa Clara, CA). Sodium and potassium were quantified by atomic absorption chromatography operated by the Department of Science Service (Bangkok, Thailand).

5.7 PHA production

5.7.1 Culture media and conditions

5.7.1.1 P(3HB) biosynthesis

M9 minimum medium (136) with or without Bacto-yeast extract (1 g/L) containing pure or crude glycerol as sole carbon source was used as culture media. To maintain the plasmids, ampicillin (100 mg/L) and ampicillin (100 mg/L) together with streptomycin (50 mg/L) were supplemented into the media for cultivation of *E. coli*-ABCs and *E. coli*-ABCsJs, respectively. The colonies on an agar plate were pre-culture in LB medium at 37° C for 15 h at 200 rpm on a rotary shaker. Then this pre-culture was inoculated into 75 or 300 mL PHA production medium which was in 250 or 1000 mL flask at an inoculums volume of 0.15 OD/mL. The cultured cells were incubated at 37° C with rotary shaking at 200 rpm. IPTG (1mM) was added to the media to induce protein expression after 3 h or 6 h of cultivation and further incubating for 24-48 h. The cultured cells were harvested by centrifugation and dried at 65° C. Cell growth was measured by OD₆₀₀ and the amount of dried cell was weighted. Protein expression was analyzed by SDS-PAGE (the method was described above).

5.7.1.2 SCL-MCL-PHAs biosynthesis

M9 minimum medium (136) with Bacto-yeast extract (1 g/L) containing pure or crude glycerol as sole carbon source was used as culture media. To maintain the plasmids, ampicillin (100 mg/L) and ampicillin (100 mg/L) together with streptomycin (50 mg/L) were supplemented into the media for cultivation of *E. coli*-ABCs and *E. coli*-ABCsJs, respectively. The colonies on an agar plate were preculture in LB medium at 37°C for 15 h at 200 rpm on a rotary shaker. Then this preculture was inoculated into 75 or 300 mL PHA production medium which was in 250 or 1000 mL flask at an inoculums volume of 0.15 OD/mL. The cultured cells were incubated at 37°C with rotary shaking at 200 rpm. Sodium dodecanoate (0.5-1.5 g/L), Brij-35 (2 g/L) (for solubilization of dodecanoate) (68), and IPTG (1 mM) were added to the medium after 3 h of cultivation, and the cells were grown for additional 24 h.
The cultured cells were harvested by centrifugation and dried at 65° C. The cell growth was measured by OD₆₀₀ and the amount of dried cell was weighted. Protein expression was analyzed by SDS-PAGE.

5.7.2 Analytical method

5.7.2.1 Fluorescent dye staining

Accumulation of P(3HB) granules inside the cells were detected by Nile Blue A staining analysis (137). Specifically, *E. coli* cells were heat-fix smeared on glass slide and stained with 1% (w/v) Nile blue A solution at 55° C for 10 min. Then, the slide was washed with tap water and 8% (v/v) acetic acid for 1 min to remove the excess stain. The prepared slide was observed under fluorescence microscope and confocal laser scanning microscope. The excitation wave length was 460 nm.

5.7.2.2 High performance liquid chromatography

5.7.2.2.1 P(3HB) quantification

To determine the quantities of P(3HB) accumulated in the *E. coli* cells, the dried cells were hydrolytically digested by sulfuric acid (138). After hydrolysis, the hydrolyzed samples were filtrated and analyzed by high performance liquid chromatography (HPLC) on an Ultimate 3000 standard LC system (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H (300 mm x 7.8 mm I.D., Bio-Rad, Hercules, CA). The samples were eluted with 10% CH₃CN in 5 mM H_2SO_4 at 65°C at a flow rate of 0.4 mL/min. Chromatogram signals were detected at 210 nm.

5.7.2.2.2 SCL-MCL-PHAs quantification and

compositions analysis

To determine the quantities and the compositions of the accumulated PHAs in *E. coli* cells, the dried cells were digested by alkaline hydrolysis with slight modification (139). Specifically, approximately 20 mg of dried cells was treated with 1 N NaOH (1 mL) at 100°C for 3 h (vortexing every 1 h) then neutralization with 1 N HCl (1 mL). The solutions were centrifuged at 21,000 *xg* for 30 min and filtrated. The filtrated samples were analyzed by HPLC on an Ultimate 3000 standard LC system (Dionex, Sunnyvale, CA) equipped with a UV detector and an Aminex HPX-87H column (300 mm x 7.8 mm I.D) (Bio-Rad, Hercules, CA) operated at 65°C at a flow rate of 0.4 mL/min. The mobile phase was 10% CH₃CN in 5 mM H₂SO₄ and chromatogram signals were detected at 210 nm.

5.7.2.2.3 Glycerol quantification

The remained glycerol concentration in the media was quantified by high performance liquid chromatography (HPLC) on a Water e2695 (Milford, MA) equipped with RI detector and a Shodex sugar SH 1011 column (8.0 mmID x 300 mmL) (New York NY) operated at 60° C at a flow rate of 0.6 mL/min. The mobile phase was 5 mM H₂SO₄.

5.7.2.3 PHAs extraction and purification

In order to determine the physical and thermal properties of polymers, the accumulated PHA in recombinant *E. coli* was extracted by solvent extraction method. The accumulated PHA in dried cell was extracted in chloroform at 80°C for 48 h after that the chloroform was evaporated to obtain PHA film. Next, the obtained PHA film was purified by dissolving in chloroform and then precipitating in 4-fold volumes of hexane or cold methanol. The white PHA powder was obtained (Fig 5.3).

Fac. of Grad. Studies, Mahidol Univ.



Dried cell

Extraction with chloroform at 80°C for 48 h

Chloroform evaporation to obtain PHA film



Precipitation with hexane or cold methanol to obtain purified PHA powder



Figure 5.3 PHA extraction and purification flowchart.

5.7.2.4 Nuclear magnetic resonance

To measure proton nuclear magnetic resonance (¹H NMR), five mg of PHA sample was dissolved in dueterated chloroform and analyzed on a Bruker AVANCE III 400 spectrometer at 400 MHz (Bruker, Helios, Singapore). To measure carbon nuclear magnetic resonance (¹³C NMR), twenty mg of PHA sample was dissolved in dueterated chloroform and analyzed on a Bruker AVANCE III 400 spectrometer at 400 MHz (Bruker, Helios, Singapore). The chemical shift of each proton and carbon resonance was assigned relying on the chemical shift of standard P(3HB) and P(3HB-*co*-3HHx) (NodaxTM).

5.7.2.5 Gel permeation chromatography

Molecular mass data on PHAs were acquired by gel permeation chromatography (GPC) analysis at 40° C using a Hewlett packard 1100 series system with a refractive index detector and a varian PLgel 10 μ m MIXED-B column (Fisher Scientific, Hampton, NH). Tetrahydrofuran was used as eluent at a flow rate of 1 mL/min. Polystyrene standards were used to construct a calibration curve.

5.7.2.6 Differential scanning calorimetry

Thermal properties were evaluated by differential scanning calorimetry (DSC) using a DSC 1 STAR^e system (Mettler Toledo, Columbus, OH). Temperature scanning was from -50°C to 200°C. The observed melting temperature $(T_{\rm m})$ was obtained from the positions of the endothermic peaks. The glass transition temperature $(T_{\rm g})$ was taken as the midpoint of heat capacity change.

5.7.2.7 Thermogravimetry analysis

The degradation temperature (T_d) was evaluated by thermogravimetry analysis (TGA) using TGA/SDTA 851^e (Mettler Toledo, Columbus, OH). Ten milligrams of polymers were heated at the rate of 20°C/min from 30°C to 500°C in a nitrogen atmosphere.

CHAPTER VI RESULTS

6.1 Establishment of PHA biosynthetic pathway in recombinant *E. coli*

In this study, we aim to investigate the biosynthesis of SCL-MCL-PHAs from glycerol by recombinant *E. coli* strains. SCL-MCL-PHAs have a wide range of applications due to their properties that can range from being thermoplastic to elastomer. These properties depend on the composition and the molar fraction between SCL-monomer and MCL-monomer. Different plastic products require different properties of plastic materials. Only a few microorganisms can produce SCL-MCL-PHAs and it is difficult to adjust the content and the compositions of copolymers produced by these bacteria as their metabolic pathways are poorly characterized and tools for genetic modification are not available. Therefore, the genetic engineering of model microorganism, *Escherichia coli*, was selected in this study. The pathway construction was divided into two main steps. First, the pathway for biosynthesis of P(3HB), employing PhaABC enzymes, was constructed in *E. coli*. Second, PhaJ was introduced into the recombinant strains to co-express with PhaABC, completing the pathway for biosynthesis of SCL-MCL-PHA copolymers.

6.1.1 Pathway design and amino acid alignment

Based on the known natural PHA biosynthesis pathways, the hybrid pathway was designed by combining two monomer-supplying pathways with one polymerization pathway. For supplying SCL-monomer, two enzymes: PhaA and PhaB of *R. eutropha* were selected to convert acetyl-CoA to (*R*)-3-hydroxybutyryl-CoA. For supplying MCL-monomers, PhaJ was selected to convert enoyl-CoA to (*R*)-3-hydroxyacyl-CoA. The designed pathway is shown in Figure 1.2.

Since variation in polymer properties leads to a wide range of applications, 3 different PhaCs and 3 different PhaJs were employed in this study to produce the different compositions of PHAs. Three different PhaCs were selected based on their different types of PHAs produced in their native strains. These are: PhaC from R. eutropha (TISTR 1095) (Pha C_{Re}) which was previously reported as SCL-PHA producing strains (140), PhaC from A. hydrophila (ATCC 7966) (PhaC_{Ah}) which was previously reported as the SCL-MCL-PHA producing strain (31) and PhaC from *P. putida* KT2440 (ATCC 47054) (PhaC2_{Pp}) which was previously reported as the MCL-PHA producing strain (141). Amino acid sequence alignment was carried out to preliminary examine their identity to each other and/or to the other well characterized enzymes. By Clustal Omega alignment, amino acid sequence of PhaC_{Re}, PhaC_{Ah}, and Pha $C2_{Pp}$ showed 36-40% identity. The conserved residues presumably forming a catalytic triad are cysteine, aspartate, and histidine (29) as indicated by the arrows in Figure 6.1. Amino acid sequence of $PhaC_{Ah}$ was elucidated by genome sequence project (70) and there is no information of heterologous expression of $PhaC_{Ah}$ in recombinant E. coli. The amino acid sequence identity of PhaC_{Ah} with the well known PhaC of A. caviae (PhaC_{Ac}) (13, 142) was as high as 92% (Fig 6.2). P. putida KT2440 have two PHA synthase enzymes (Pha $C1_{Pp}$ and Pha $C2_{Pp}$). Both of them showed high expression level when P. putida KT2440 were grown under the conditions for PHA production suggested that $PhaC1_{Pp}$ and $PhaC2_{Pp}$ involved in PHA biosynthesis (62). However, only PhaC1 has been cloned and characterized in recombinant E. coli (67, 143). There is no information of heterologous expression of $PhaC2_{Pp}$ in recombinant E. coli. Amino acid sequence alignment of $PhaC1_{Pp}$ and $PhaC2_{Pp}$ showed that their identity was 54% (Fig 6.3). On the other hand, three different PhaJ enzymes (PhaJ1_{Pp}, PhaJ4_{Pp}, and PhaJ_{Ah}) which have broad substrate specificity were selected. PhaJ1_{Pp} and PhaJ4_{Pp} were from P. putida KT2440 which were identified by homology search of PhaJ1-4 from P. aeruginosa against DNA genome sequence of P. putida KT2440 (69), whereas $PhaJ_{Ah}$ from A. hydrophila (ATCC 7966) has not been characterized previously. Clustal Omega alignment showed that $PhaJ1_{Pp}$, $PhaJ4_{Pp}$, and $PhaJ_{Ah}$ have 26-36% identity of their amino acid sequences (Fig 6.4). The residues forming a catalytic dyad were aspartate and histidine identified based on the crystal structure of A. caviae (144). Amino acid sequence of $PhaJ_{Ah}$ was compared with that of PhaJ from A. caviae which is well characterized and they showed identity as high as 81% (Fig 6.5).

C2Pp CRe CAh	TLPATRMNVQNAILGLRGR MATGKGAAASTQEGKSQPFKVTPGPFDPATWLEWSRQWQGTEGNGHAAASGIPGL MSQSSYGPLFEALAHYNDKLLDMAKAQTERTAQALLQTNL :: ::	29 55 40
C2Pp CRe CAh	DLLSTLRNVGRHGLRHPLHTAHHLLALGGQLGRVMLGDTPYQPNP DALAGVKIAPAQLGDIQQRYMKDFSALWQ-AMAEGKAEATGPL DDLGQVLEQGSQQPWQLINAQMNWWQDQLKLMQHTLLKSAGQQSEPLIAPER * * : * :	74 97 92
C2Pp CRe CAh	RDARFSDPTWSQNPFYRRGLQAYLAWQKQTRQWIDESH-LNDDDRARAHFLFNLINDALA HDRRFAGDAWRTNLPYRFAAAFYLLNARALTELADAVEA-DAKTRQRIRFAISQWVDAMS SDRRFKADAWSEQPIYDYLKQSYLLTAKHLLASVDALEGVPQKSRERLRFFTRQYINAMA * ** :* : * :* :* :* :* :* :* :*	133 156 152
C2Pp CRe CAh	PSNSL-LNPLAVKELFNTGGQSLVRGVAHLLDDLRHNDGLPRQVDERAFEVGVNLAAT PANFLATNPEAQRLLIESGGESLRAGVRNMMEDLTRGKISQTDESAFEVGRNVAVT PSNFLATNPELLKLTLESDGQNLVRGLALLAEDLERSADQLNIRLTDESAFELGRDLALT *:* * ** : ::: *:.* *: :**:	190 212 212
C2Pp CRe CAh	PGAVVFRNELLELIQYSPMSEKQHARPLLVVPPQINKFYIFDLSATNSFVQYMLKSGLQV EGAVVFENEYFQLLQYKPLTDKVHARPLLMVPPCINKYYILDLQPESSLVRHVVEQGHTV PGRVVQRTELYELIQYSPTTETVARTPVLIVPFINKYYILDMRPQNSLVAWLVAQGQTV * *** :*:**.* ::. *:*:*** ***:**:*: ***	250 272 272
C2Pp CRe CAh	FMVSWRNPDPRHREWGLSSYVQ-ALEEALNACRSISGNRDPNLMGACAGGLTMAALQGHL FLVSWRNPDASMAGSTWDDYIEHAAIRAIEVARDISGQDKINVIGFCVGGTIVSTALAVL FMISWRNPGPAQADIDLDDYVVDGVIAALDGVEAATGEREVHGIGYCIGGTALSLAMGWL *::*****	309 332 332
C2Pp CRe CAh	QAKKQLRRVRSATYLVSLLDSKFESPASLFADEQTIEAAKRRSYQRGVLDGGEVA AARGE-HPAASVTLLTTLLDFADTGILDVFVDEGHVQLREATLGGGAGAPCALLRGLELA AARRQKQRVRSATLFTTLLDFSQPGELGIFIHEPIIAALEAQNEAKGIMDGRQLA *: : : . *.* :.:*** : : : : : : : : : :	364 391 387
C2Pp CRe CAh	RIFAWMRPNDLIWNYWVNNYLLGKTPPAFDILYWNADSTRLPAALHGDLLEF-FKLNPLT NTFSFLRPNDLVWNYVVDNYLKGNTPVPFDLLFWNGDATNLPGPWYCWYLRHTYLQNELK VSFSLLRENSLYWNYYIDSYLKGQSPVAFDLLHWNSDSTNVAGKTHSSLLRRLYLENQLG *: :* *.* *** ::. * *:: * *:* *:* *:* *:	423 451 447
C2Pp CRe CAh	YASGLEVCGTFIDLQQVNIDSFTVAGSNDHITPWDAVYRSALLLGGERRFVLANSGHIQS VPGKLTVCGVPVDLASIDVPTYIYGSREDHIVPWTAAYASTALLANKLRFVLGASGHIAG -KGELKIRHTRIDLGKVKTPVLLVSAMDDHIALWQGTWQGMKLFGGEQRFILAESGHIAG * : . :** .:. **** . *: *:. *:. ****	483 511 506
C2Pp CRe CAh	IINPPGNPKAYYLANPKLSSDPRAWFHDAKRSEGSWWPLWLEWITARSGLLKAPRTELGN VINPPAKNKRSHWTNDALPESPQQWLAGAIEHHGSWWPDWTAWLAGQAGAKRAAPANYGN IVNPPDANKYGFWQNDAEAISPEAWLAGASHTPGSWWPQMAAFIQSREEDPAPLPARI ::*** * * * * * * * * * * * ***** :: ::	543 571 564
C2Pp CRe CAh	ATYPPLGPAPGTYVLTR 560 ARYRAIEPAPGRYVKAKA 589 -PSEGLEAAPGSYVKVRLNPVFAQTPEEEPA 594 : *** ** .: 594	

Figure 6.1 Amino acid sequence alignment of PhaCs.

PhaC_{Ah} (CAh), PhaC_{Re} (CRe), and PhaC2_{Pp} (C2Pp) have 594, 589, and 560 amino acids, respectively. The gene IDs of PhaC_{Ah}, PhaC_{Re}, and PhaC2_{Pp} are 4488480, 4250156, and 1041897, respectively. Their sequences were aligned using Clustal Omega (<u>www.ebi.ac.uk/Tools/msa/clustalo/</u>). They share 36-40% identity. The box indicates the putative lipase box. The arrows indicate the conserved residues involved in catalysis (4). A colon (:) indicates conservation between groups of strongly similar properties. A dot (.) indicates identical residues. Numbers indicate the position of amino acids in the respective proteins.

Chitwadee Phithakrotchanakoon

CAh CAc	MSQSSYGPLFEALAHYNDKLLDMAKAQTERTAQALLQTNLDDLGQVLEQGSQQPWQLINA MSQFSYGPLFEALAHYNDKLLAMAKAQTERTAQALLQTNLDDLGQVLEQGSQQPWQLIQA *** *********************************	60 60
CAh CAc	QMNWWQDQLKLMQHTLLKSAGQQSEPLIAPERSDRRFKADAWSEQPIYDYLKQSYLLTAK QMNWWQDQLKLMQHTLLKSAGQPSEPVITPERSDRRFKAEAWSEQPIYDYLKQSYLLTAR ************************************	120 120
CAh CAc	HLLASVDALEGVPQKSRERLRFFTRQYINAMAPSNFLATNPELLKLTLESDGQNLVRGLA HLLASVDALEGVPQKSRERLRFFTRQYVNAMAPSNFLATNPELLKLTLESDGQNLVRGLA ************************************	180 180
CAh CAc	LLAEDLERSADQLNIRLTDESAFELGRDLALTPGRVVQRTELYELIQYSPTTETVARTPV LLAEDLERSADQLNIRLTDESAFELGRDLALTPGRVVQRTELYELIQYSPTTETVGKTPV ************************************	240 240
CAh CAc	LIVPPFINKYYILDMRPQNSLVAWLVAQGQTVFMISWRNPGPAQADIDLDDYVVDGVIAA LIVPPFINKYYIMDMRPQNSLVAWLVAQGQTVFMISWRNPGVAQAQIDLDDYVVDGVIAA ************	300 300
CAh CAc	LDGVEAATGEREVHGIGYCIGGTALSLAMGWLAARRQKQRVRSATLFTTLLDFSQPGELG LDGVEAATGEREVHGIGYCIGGTALSLAMGWLAARRQKQRVRTATLFTTLLDFSQPGELG ***********************************	360 360
CAh CAc	IFIHEPIIAALEAQNEAKGIMDGRQLAVSFSLLRENSLYWNYYIDSYLKGQSPVAFDLLH IFIHEPIIAALEAQNEAKGIMDGRQLAVSFSLLRENSLYWNYYIDSYLKGQSPVAFDLLH ***********************************	420 420
CAh CAc	WNSDSTNVAGKTHSSLLRRLYLENQLGKGELKIRHTRIDLGKVKTPVLLVSAMDDHIALW WNSDSTNVAGKTHNSLLRRLYLENQLVKGELKIRNTRIDLGKVKTPVLLVSAVDDHIALW ************************************	480 480
CAh CAc	QGTWQGMKLFGGEQRFILAESGHIAGIVNPPDANKYGFWQNDAEAISPEAWLAGASHTPG QGTWQGMKLFGGEQRFLLAESGHIAGIINPPAANKYGFWHNGAEAESPESWLAGATHQGG *****************	540 540
CAh CAc	SWWPQMAAFIQSREEDPAPLPARIPSEGLEAAPGSYVKVRLNPVFAQTPEEEPA 594 SWWPEMMGFIQNRDEGSEPVPARVPEEGLAPAPGHYVKVRLNPVFACPTEEDAA 594 ****:* *** *:* *:****	

Figure 6.2 Amino acid sequence alignment of PhaC from *A. hydrophila* ATCC 7966 and from *A. caviae*.

Both PhaC_{*Ah*} from *A. hydrophila* ATCC 7966 (CAh) and PhaC_{*Ac*} from *A. caviae* (CAc) have 594 amino acids. The sequence IDs of PhaC_{*Ah*} and PhaC_{*Ac*} are 4488480 and 325300783, respectively. Their sequences were aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). They share 92% identity. A colon (:) indicates conservation between groups of strongly similar properties. A dot (.) indicates identical residues. Numbers indicate the position of amino acids in the respective proteins.

C1Pp C2Pp	MSNKNNDELQRQASENTLGLNPVIGIRRKDLLSSARTVLRQAVRQPLHSAKHVAHFGLEL MTDKPAKGSTTLPATRMNVQNAILGLRGRDLLSTLRNVGRHGLRHPLHTAHHLLALGGQL *::* : : : : : : : : : : : : : : : : :	60 60
C1Pp C2Pp	KNVLLGKSSLAPDSDDRRFNDPAWSNNPLYRRYLQTYLAWRKELQDWVSSSDLSPQDISR GRVMLGDTPYQPNPRDARFSDPTWSQNPFYRRGLQAYLAWQKQTRQWIDESHLNDDDRAR .*:**.: *: * **.**:**:*** **:*****:*: ::*:.*.*.*	120 120
C1Pp C2Pp	GQFVINLMTEAMAPTNTLSNPAAVKRFFETGGKSLLDGLSNLAKDMVNNGGMPSQVNMDA AHFLFNLINDALAPSNSLLNPLAVKELFNTGGQSLVRGVAHLLDDLRHNDGLPRQVDERA .:*::**:.:*:**:*:*********************	180 180
C1Pp C2Pp	FEVGKNLGTSEGAVVYRNDVLELIQYSPITEQVHARPLLVVPPQINKFYVFDLSPEKSLA FEVGVNLAATPGAVVFRNELLELIQYSPMSEKQHARPLLVVPPQINKFYIFDLSATNSFV **** **.:: ****:***::******::*: ********	240 240
C1Pp C2Pp	RFCLRSQQQTFIISWRNPTKAQREWGLSTYIDALKEAVDAVLSITGSKDLNMLGACSGGI QYMLKSGLQVFMVSWRNPDPRHREWGLSSYVQALEEALNACRSISGNRDPNLMGACAGGL :: *:* *.*::***** :******:*:**:*********	300 300
C1Pp C2Pp	TCTALVGHYAAIGE-NKVNALTLLVSVLDTTMDNQVALFVDEQTLEAAKRHSYQAGVLEG TMAALQGHLQAKKQLRRVRSATYLVSLLDSKFESPASLFADEQTIEAAKRRSYQRGVLDG * :** ** * : ::::: * ***:**:::: ::**.***:********	359 360
С1Рр С2Рр	SEMAKVFAWMRPNDLIWNYWVNNYLLGNEPPVFDILFWNNDTTRLPAAFHGDLIEMFKSN GEVARIFAWMRPNDLIWNYWVNNYLLGKTPPAFDILYWNADSTRLPAALHGDLLEFFKLN .*:*::********************************	419 420
С1Рр С2Рр	PLTRPDALEVCGTAIDLKQVKCDIYSLAGTNDHITPWPSCYRSAHLFGGKIEFVLSNSGH PLTYASGLEVCGTPIDLQQVNIDSFTVAGSNDHITPWDAVYRSALLLGGERRFVLANSGH ********* ***:**: * :::**:******* : ***** ::****	479 480
С1Рр С2Рр	IQSILNPPGNPKARFMTGADRPGDPVAWQENAIKHADSWWLHWQSWLGERAGALKKAPTR IQSIINPPGNPKAYYLANPKLSSDPRAWFHDAKRSEGSWWPLWLEWITARSGLLKAPRTE ****:******* :::** ** .:* : *** * .*: *:* **	539 540
C1Pp C2Pp	LGNRTYAAGEASPGTYVHER 559 LGNATYPPLGPAPGTYVLTR 560 *** ** :***** *	

Figure 6.3 Amino acid sequence alignment of PhaC1 and PhaC2 from *P. putida* KT2440.

PhaC1_{*Pp*} (C1Pp) and PhaC2_{*Pp*} (C2Pp) have 559 and 560 amino acids, respectively. The gene IDs of PhaC1_{*Pp*} and PhaC2_{*Pp*} are 1041855 and 1041897, respectively. Their sequences were aligned using Clustal Omega (<u>www.ebi.ac.uk/Tools/msa/clustalo/</u>). They share 54% identity. A colon (:) indicates conservation between groups of strongly similar properties. A dot (.) indicates identical residues. Numbers indicate the position of amino acids in the respective proteins.

J4Pp J1Pp JAh	MPHVPVTELSQYVGKELGHSEWLKIDQQRINLFAEATGDFQFIHVDPEKAAKTPFGGT MSQVTNTPYEALEVGQKAEYKKSVEERDIQLFAAMSGDHNPVHLDAEFAAKSMFRER MSMPPFEVGQQASLTKRFGAAEVEAFAGLSEDFNPLHLDPAFAATTPFERP *:*:. :: ** : *.: :*:* **.: *	58 57 51
J4Pp J1Pp JAh	IAHGFLTLSLIPKLIEDILVLPQGLKMVVNYGLDSVRFIQPVKVDSRVRLKVKLGEVVEK IAHGMFSGALISAAVACTLPGPGTIYLGQQMSFQKPVKIGDTLTVRLEILEKLPK IVHGMLLASLFSGLLGQQLPGKGTVYLGQSLAFKQPVFVGDEVTAEVEIIALRSD *.**:: :*: : * * ::: *:::::::::	118 112 106
J4Pp J1Pp JAh	KPGQWLLKAIATLEIEGEEKPAYIAESLSLCFV 151 F-KVRIATNVYNQNDELVVAGEAEILAPRKQQTVELVSPPNFVAS 156 KPIITLATRILTATGAIAVSGEAVVKFG 134 : **	

Figure 6.4 Amino acid sequence alignment of PhaJs.

PhaJ_{Ah} (JAh), PhaJ1_{Pp} (J1Pp), and PhaJ4_{Pp} (J4Pp) have 134, 156, and 151 amino acids. The gene IDs of PhaJ_{Ah}, PhaJ1_{Pp} and PhaJ_{4Pp} are 1046178, 1045908, and 4488481, respectively. Their sequences were aligned using Clustal Omega (<u>www.ebi.ac.uk/Tools/msa/clustalo/</u>). They share 26-36% identity. The arrows indicate the conserved residues involved in catalysis (12). A colon (:) indicates conservation between groups of strongly similar properties. A dot (.) indicates identical residues. Numbers indicate the position of amino acids in the respective proteins.

JAh	MSMPPFEVGQQASLTKRFGAAEVEAFAGLSEDFNPLHLDPAFAATTPFERPIVHGMLLAS	60
JAC	MSAQSLEVGQKARLSKRFGAAEVAAFAALSEDFNPLHLDPAFAATTAFERPIVHGMLLAS	60
	** •****•* *•******* *** _• *************	
JAh	LFSGLLGQQLPGKGTVYLGQSLAFKQPVFVGDEVTAEVEIIALRSDKPIITLATRILTAT	120
JAC	$\tt LFSGLLGQQLPGKGSIYLGQSLSFKLPVFVGDEVTAEVEVTALREDKPIATLTTRIFTQG$	120

JAh	GAIAVSGEAVVKFG 134	
JAC	GALAVTGEAVVKLP 134	
	** ** ******	

Figure 6.5 Amino acid sequence alignment of PhaJ from *A. hydrophila* ATCC 7966 and from *A. caviae*.

Both PhaJ_{Ah} (JAh) and PhaJ_{Ac} (JAc) have 134 amino acids. The sequence IDs of PhaJ_{Ah} and PhaJ_{Ac} are 4488481 and 2335048, respectively. Their sequences were aligned using Clustal Omega (<u>www.ebi.ac.uk/Tools/msa/clustalo/</u>). They share 81% identity. A colon (:) indicates conservation between groups of strongly similar properties. A dot (.) indicates conservation between groups of weakly similar properties. An asterisk (*) indicates identical residues. Numbers indicate the position of amino acids in the respective proteins.

Fac. of Grad. Studies, Mahidol Univ.

6.1.2 Plasmid construction

To establish the PHA biosynthetic pathway, two sets of recombinant plasmids were constructed. The first one was pETDuet-1 vector harboring *phaA*, *phaB*, and *phaC*. The second one was pCDFDuet-1 vector harboring *phaJ*. As three types of phaC and three types of phaJ were used, six resulting recombinant plasmids namely pETDuet-*ABC_{Re}*, pETDuet-*ABC_{Ah}*, pETDuet-*ABC2_{Pp}*, pCDFDuet-*J1_{Pp}*, pCDFDuet-*J4_{Pp}*, and pCDFDuet-*J_{Ah}* were constructed (Fig 5.2). All genes were PCR amplified from the genomic DNA of native PHA producing strains. The thick bands of extracted genomic DNA are found at the position higher than 23 kb (Fig 6.6). The PCR amplified fragments were purified by excising only the target PCR bands of *phaAB_{Re}*, *phaC_{Re}*, *phaC_{Ah}*, and *phaC2_{Pp}* (Fig 6.7). The PCR amplified fragment of *phaJ1_{Pp}*, *phaJ4_{Pp}*, and *phaJ_{Ah}* are shown in Figure 6.8.



Figure 6.6 Genomic DNA extracted from wild type PHA-producing bacteria.

Agarose gel electrophoresis of genomic DNA from wild type PHAproducing bacteria using SDS-based DNA extraction method with ethylenediaminetetraacetic acid (EDTA) and phenol chloroform. Lane M is λ /*Hind*III marker. Lanes 1-3 are extracted genomic DNA from *R. eutropha*, *A. hydrophila*, and *P. putida* respectively.



Figure 6.7 PCR amplification of β-ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*), and PHA synthase (*phaC*) genes from genomic DNA of wild type PHA-producing bacteria.

Agarose gel electrophoresis of the DNA fragment from PCR amplification of *phaA*, *phaB*, and *phaC* purified genomic DNA. Lane M is 1 kb DNA marker. Lane 1, *phaA-phaB* from *R. eutropha* (the expected size is ~2.0 kb); lane 2, *phaC* of *R. eutropha* (the expected size is ~1.8 kb); lane 3, *phaC* of *A. hydrophila* (the expected size is ~1.8 kb); lane 4, *phaC* of *P. putida* (the expected size is ~1.7 kb).



Figure 6.8 PCR amplification of (*R*)-specific enoyl-CoA hydratase (*phaJ*) genes from genomic DNA of wild type PHA-producing bacteria.

Agarose gel electrophoresis of the DNA fragment from PCR amplification. Lane M is 100 bp DNA ladder plus marker. Lane 1, *phaJ*1 from *P. putida* (the expected PCR fragment is 0.48 kb); lane 2, *phaJ*4 from *P. putida* (the expected PCR fragment is 0.46 kb); lane 3, *phaJ* from *A. hydrophila* (the expected PCR fragment is approximately 0.4 kb).

6.1.3 Protein expression

E. coli BL21(DE3) was used as a host for protein expression and PHA production. Three recombinant E. coli strains harboring phaA, phaB, and phaC namely E.coli-AB_{Re}C_{Re}, E.coli-AB_{Re}C_{Ah}, and E.coli-AB_{Re}C2_{Pp} were tested for their ability to co-express PhaABC in LB medium. The heterologous protein expression of β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) proteins in E. coli BL21(DE3) is shown (Fig 6.9). The results showed that all target recombinant proteins were expressed under the condition used. Compared to the *E. coli* strain harboring the empty vector pETDuet-1, $PhaA_{Re}$, $PhaC_{Re}$, $PhaC_{Ah}$, and $PhaC_{2Pp}$ were clearly expressed from the recombinants. However, the expression level of each protein was different. From SDS-PAGE analysis, the expression of $PhaB_{Re}$ was not clear but its expression was confirmed by western blot (Fig 6.9B). The expression of PhaJ in E. coli-J1_{Pp}, E. coli-J4_{Pp}, and E. coli-J_{Ah} were verified. SDS-PAGE and western blot analysis using anti-His₆ showed that all three PhaJs were expressed (Fig 6.10). Although the expression of $PhaJ4_{Pp}$ and PhaJ_{Ah} could not be clearly seen from SDS-PAGE, they were detectable using western blot.



Figure 6.9 Heterologous protein expression of β-ketothiolase (PhaA), NADPHdependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) proteins in *E. coli* BL21(DE3).

(A) SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}, *E. coli*-ABC_{*Ah*}, and *E. coli*-ABC2_{*Pp*} cultured in LB medium and induced with 1 mM IPTG for 3 h. (B) western blot analysis using anti-polyHistidine monoclonal antibody. Lane M is Prestained Protein molecular weight marker. Lanes 1-3 are the proteins from *E. coli*-ABC_{*Re*}, *E. coli*-ABC_{*Ah*}, and *E. coli*-ABC2_{*Pp*}, respectively. Ctrl is proteins from *E. coli*-pETDuet-1 (control). The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, and PhaC2_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, and 64 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.



Figure 6.10 SDS-PAGE and western blot analysis of heterologous protein expression of (*R*)-specific enoyl-CoA hydratase (PhaJ) proteins in *E. coli* BL21(DE3).

(A) is *E. coli*-J1_{*Pp*}, (B) is *E. coli*-J4_{*Pp*} and (C) is *E. coli*-J_{*Ah*}. Cells were cultured in LB medium and induced with 1 mM IPTG for 3 h. Western blot analysis was performed using anti-polyHistidine monoclonal antibody. Lanes M1 and M2 are Unstained and Prestained Protein molecular weight marker. Ctrl is proteins from *E. coli*-PCDFDuet-1 (control). PhaJ1_{*Pp*}, PhaJ4_{*Pp*}, and PhaJ_{*Ah*} proteins are indicated by arrows. The predicted protein molecular weight of PhaJ1_{*Pp*}, PhaJ4_{*Pp*}, and PhaJ_{*Ah*} were 19 kDa, 18 kDa and 16 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.

To establish the hybrid pathway for biosynthesis of SCL-MCL-PHAs in *E. coli*, the recombinant plasmids, pETDuet-*ABC*s and pCDFDuet-*J*s, were co-transformed into *E. coli* BL21(DE3). Nine recombinant strains namely, *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC2_{*Pp*}J1_{*Pp*}, *E. coli*-ABC2_{*Pp*}J4_{*Pp*}, *and E. coli*-ABC2_{*Pp*}J4_{*Pp*}, *E. coli*-ABC2_{*Pp*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J_{*Ah*} were generated. Co-expression of PhaA, PhaB, PhaC and PhaJ was verified. Figure 6.11 showed SDS-PAGE and western blot analysis of proteins in recombinant *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *E. coli*-ABC2_{*Pp*}J4_{*Pp*}. Figure 6.12 showed SDS-PAGE and western blot analysis of proteins in recombinant *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *and E. coli*-ABC2_{*Pp*}J4_{*Pp*}. Figure 6.13 showed SDS-PAGE and western blot analysis of proteins in recombinant *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *And E. coli*-ABC2_{*Pp*}J4_{*Pp*}. Figure 6.13 showed SDS-PAGE and western blot analysis of proteins in recombinant *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *B. coli*-ABC2_{*Pp*}J4_{*Pp*}. Figure 6.13 showed SDS-PAGE and western blot analysis of proteins in recombinant *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *B. coli*-ABC2_{*Pp*}J_{*Ah*}. The results showed that all recombinant proteins were co-expressed in *E. coli*.}}}}

(A)



Figure 6.11 Co-expression of β -ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and (*R*)-specific enoyl-CoA hydratase (PhaJ1_{*Pp*}) proteins in *E. coli* BL21(DE3).

(A) SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J1_{*Pp*} cultured in LB medium and induced with 1 mM IPTG for 3 h. (B) Western blot analysis using anti-polyHistidine monoclonal antibody. Lane M is Prestained Protein molecular weight marker. Lanes 1-3 are the proteins from *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J1_{*Pp*}, respectively. Ctrl is proteins from *E. coli*-pETDuet-1+pCDFDuet-1 (control). The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*} and PhaJ1_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 19 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.

(B)



Figure 6.12 Co-expression of β -ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and (*R*)-specific enoyl-CoA hydratase (PhaJ4_{*Pp*}) proteins in *E. coli* BL21(DE3).

(A) SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J4_{*Pp*} cultured in LB medium and induced with 1 mM IPTG for 3 h. (B) western blot analysis using anti-polyHistidine monoclonal antibody. Lane M is Prestained Protein molecular weight marker. Lanes 1-3 are the proteins from *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J4_{*Pp*} respectively. Ctrl is proteins from *E. coli*-pETDuet-1+pCDFDuet-1 (control). The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*} and PhaJ4_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 18 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.



Figure 6.13 Co-expression of β -ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), PHA synthase (PhaC) and (*R*)-specific enoyl-CoA hydratase (PhaJ_{Ah}) proteins in *E. coli* BL21(DE3).

(A) SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, and *E. coli*-ABC2_{*Pp*}J_{*Ah*} cultured in LB medium and induced with 1 mM IPTG for 3 h. (B) Western blot analysis using anti-polyHistidine monoclonal antibody. Lane M is Prestained Protein molecular weight marker. Lanes 1-3 are the proteins from *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, and *E. coli*-ABC2_{*Pp*}J_{*Ah*} respectively. Ctrl is proteins from *E. coli*-pETDuet-1+pCDFDuet-1 (control). The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*} and PhaJ_{*Ah*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 16 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.

6.2 Using pure glycerol as a carbon source for PHAs biosynthesis

6.2.1 P(3HB) biosynthesis

6.2.1.1 Effect of different PhaC types on growth and P(3HB) biosynthesis by *E. coli*-ABCs

The ability of recombinant E. coli harboring PhaA, PhaB, and PhaCs (E. coli-ABC_{Re}, E. coli-ABC_{Ah}, and E. coli-ABC_{2Pp}) to synthesize P(3HB) by using pure glycerol as the sole carbon source was investigated by cultivating the recombinant strains in M9 medium containing glycerol. Their growth curve profiles were determined (Fig 6.14). All strains reached the log phase and stationary phase at 3 h and 15 h after inoculation, respectively. IPTG (1mM) was added to the culture media for induction of recombinant proteins at 6 h after inoculation (early log phase). The expression of recombinant proteins from each recombinant strains are shown (Fig 6.15). Accumulation of P(3HB) granules inside the cells were detected by Nile Blue A staining analysis. The stained cells were observed under Fluorescence Microscope Olympus (UIS Fluorescence) at 400x (Fig 6.16). The result showed that E. coli-ABC_{Re} and E. coli-ABC_{Ah} emitted orange fluorescence whereas E. coli- $ABC2_{Pp}$ did not emit orange fluorescence suggesting that E. coli-ABC_{Re} and E. coli- ABC_{Ah} can accumulate P(3HB) granules inside the cells under conditions used. Moreover, the round orange granules of P(3HB) accumulated inside of E. coli-ABC_{Re} and E. coli-ABC_{Ah} were clearly shown by observing under Confocal Laser Scanning Microscope model Olympus FV1000 at 1000x (Fig 6.17). To quantitate the P(3HB) content, the dried cells of each recombinant were digested by H₂SO₄ to its derivative monomer and subjected to HPLC analysis. The results showed that different sources of PhaC gave different level of P(3HB) (Table 6.1). E. coli-ABC_{Ah}, E. coli-ABC_{Re}, and E. coli-ABC2_{Pp} accumulated P(3HB) at 30 wt%, 24 wt% and 2 wt% content of dry cell weight, respectively. Since E. coli-ABC_{Ah} was able to produce the highest amount of P(3HB), therefore this recombinant strain was then used for further investigating the effect of some cultivation parameters on the production of P(3HB).

Fac. of Grad. Studies, Mahidol Univ.



Figure 6.14 Growth profiles of recombinant *E. coli*-ABCs strains cultured in minimum medium (M9) containing 2% (v/v) glycerol as a sole carbon source. Result is the average value and error bar represents standard deviation for triplicate experiments.



Figure 6.15 Protein expression profiles of *E. coli*-ABC_{*Re*}, *E. coli*-ABC_{*Ah*}, and *E. coli*-ABC_{*Pp*} analyzed by SDS-PAGE.

SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}, *E. coli*-ABC_{*Ah*}, and *E. coli*-ABC2_{*Pp*} cultured in M9 medium containing 2% (v/v) glycerol as a sole carbon source. The cells were induced with 1 mM IPTG after 6 h inoculation and harvested after 24 h. Lane M is Unstained Protein molecular weight marker. Lanes 1-2, 3-4, 5-6, and 7-8 are the proteins from *E. coli*-ABC_{*Re*}, *E. coli*-ABC_{*Ah*}, *E. coli*-ABC2_{*Pp*}, and *E. coli*-pETDuet-1, respectively. The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, and PhaC2_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, and 64 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.



Figure 6.16 Nile blue A fluorescent dye staining of P(3HB) granules in recombinant *E. coli*-ABCs.



Figure 6.17 Nile blue A fluorescent dye staining of P(3HB) granules in recombinant *E. coli*-ABCs as observed by confocal laser scanning microscope.

(A) *E. coli*-ABC_{*Re*}, (B) *E. coli*-ABC_{*Ah*}, (C) *E. coli*-ABC2_{*Pp*}, and (D) *E. coli*pETDuet-1 (control). The recombinant *E. coli* were cultured in M9 containing 2% (v/v) glycerol as sole carbon source and induced with 1 mM IPTG for 24 hr. The micrographs were taken under Fluorescence Microscope Olympus (UIS Fluorescence) at 400x (Fig 6.16) and under Confocal Laser Scanning Microscope model Olympus FV1000 at 1000x (Fig 6.17).

Strains	CDW (g/L)	P(3HB) content (wt% of CDW)	P(3HB) (mg/L)
E. coli-ABC _{Ah}	1.23 ± 0.05	30.4 ± 1.61	371.8 ± 32.64
$E. \ coli-ABC_{Re}$	1.08 ± 0.05	24.4 ± 0.95	264.9 ± 21.16
E. coli-ABC2 _{Pp}	0.95 ± 0.01	1.8 ± 0.23	13.5 ± 2.37
E. coli-pETDuet-1	0.82 + 0.002		
(control)	0.82 ± 0.003	-	-

Table 6.1 P(3HB) biosynthesis in recombinant E. coli harboring phaABCs[reproduced from (19) with permission from JSBA].

Cells were cultivated at 37°C in M9 minimum medium containing 2% (v/v) glycerol as the sole carbon source. After 6 h of cultivation, IPTG (1mM) was added and cells were grown for additional 24 h. Results are the average \pm standard deviations from three independent experiments. CDW, cell dry weight; -, not detectable.

6.2.1.2 Effect of glycerol concentration on growth and P(3HB) biosynthesis by *E. coli*-ABC_{Ah}

The effect of glycerol concentration on the growth of *E. coli*-ABC_{*Ah*} was investigated. Glycerol concentrations varying from 1% to 15% (v/v) were used as a carbon source for cultivating *E. coli*-ABC_{*Ah*}. The cultured cells were collected at various intervals to measure the OD₆₀₀ (Fig 6.18). The growth profiles of *E. coli*-ABC_{*Ah*} cultured in the medium containing 1%, 2%, or 5% (v/v) glycerol were similar, having their maximal OD₆₀₀ over 2.0. But in 10% and 15% (v/v) glycerol, the growths of *E. coli*-ABC_{*Ah*} were slower and their maximum OD₆₀₀ were around 1.5. Therefore, glycerol concentrations up to only 5% (v/v) were used as a carbon source to evaluate their effect on the ability to produce P(3HB) of *E. coli*-ABC_{*Ah*}. After 3 h inoculation, the cultured cells were induced by 1 mM IPTG and cultured for further 24 h. Cell dry weight, P(3HB) content, and the remaining glycerol in the supernatant after cell harvested were determined (Table 6.2). The result showed that the maximal cell dry weight and the amount of P(3HB) were obtained from 1% (v/v) glycerol.

However, only a slight decrease of cell dry weight and the amount of P(3HB) were found with increasing glycerol concentration. Moreover, it was found that glycerol still remained in the culture media after cultivation for 27 h in all concentrations used.



Figure 6.18 Effect of glycerol concentration on the growth profiles of *E. coli*-ABC_{*Ah*}.

E. coli-ABC_{*Ah*} was cultured in M9 minimum medium supplemented with yeast extract with 1%, 2%, 5%, 10%, and 15% (v/v) glycerol as a carbon source. The cultured cells were collected at 0, 3, 5, 11, 24, 30, 35, 51, and 58 h after inoculation and OD_{600} of the cultured media were measured. Result is the average value and error bar represents standard deviation for triplicate experiment.

Glycerol	CDW	P(3HB) content	P(3HB)	Remaining
(% v/v)	(g/L)	(wt% of CDW)	(g/L)	glycerol (% v/v)
1	1.6 ± 0.10	23.2 ± 0.92	0.37 ± 0.04	0.2 ± 0.03
2	1.4 ± 0.10	21.7 ± 0.92	0.30 ± 0.01	1.3 ± 0.02
3	1.4 ± 0.10	18.3 ± 1.33	0.25 ± 0.01	2.4 ± 0.06
5	1.3 ± 0.15	19.9 ± 0.56	0.27 ± 0.02	4.3 ± 0.04

Table 6.2 Effect of glycerol concentration on P(3HB) biosynthesis.

Cells were cultivated at 37° C in M9 minimum medium containing glycerol as the sole carbon source supplemented with 1 g/L Bacto-yeast extract. After 3 h of cultivation, IPTG (1mM) was added and cells were grown for an additional 24 h. Results are the average \pm standard deviations from triplicate experiment. CDW, cell dry weight.

6.2.1.3 Effect of carbon and nitrogen source on growth and P(3HB) biosynthesis by *E. coli*-ABC_{*Ah*}

P(3HB) production using glycerol as a carbon source was compared to glucose as a sole carbon source. Moreover, the effect of adding yeast extract into the culture media was also evaluated. To investigate the effect of carbon and nitrogen source on the growth of *E. coli*-ABC_{*Ah*}, the cells were cultured in the media containing glycerol or glucose at 2% (w/v) as a carbon source with or without yeast extract. The cultured cells were collected every 3 h until 33 h to measure the OD₆₀₀ and pH. In all culture media, the highest cell density was equal at 30 h. By using glycerol as a carbon source, cells grew slower than that using glucose. When yeast extract was supplemented in the culture media with either glycerol or glucose as a carbon source, cell grew rapidly, had shorter lag phase and entered into the stationary phase within 14 h. Whereas when the cells were grown in the media without yeast extract, they grew slower, with longer lag phase and entered into the stationary phase after 14 h (Fig 6.19). The pH of the culture media decreased rapidly when cells were grown in glucose within 14 h. At 30 h, pH was drop to 5.0 and 4.5 when cells were grown in glycerol and glucose, respectively (Fig 6.20).



Figure 6.19 Effect of carbon and nitrogen source on the growth profiles of *E. coli*-ABC_{*Ah*}.



Figure 6.20 Effect of carbon and nitrogen source on pH profiles of E. coli-ABC_{Ah}.

E. coli-ABC_{*Ah*} was cultured in four types of media which were M9+glucose+yeast extract, M9+glycerol+yeast extract, M9+glucose, and M9+glycerol. The cultured cells of *E. coli*-ABC_{*Ah*} were collected at the interval time to measure the OD₆₀₀ and pH. Result is the average value and error bar represents standard deviation for triplicate experiment.

Next, the effect of carbon and nitrogen source on the ability of *E. coli*-ABC_{*Ah*} to synthesize P(3HB) was investigated. *E. coli*-ABC_{*Ah*} was cultured in three different media which were M9 medium containing glucose and yeast extract, glycerol and yeast extract, and glycerol only. The cells were induced with 1 mM IPTG at the early log phase. Based on the growth profiles (Fig 6.19), the cells cultured in media with yeast extract were induced at 3 h after inoculation and continued culture for additional 24 h and 48 h. While the cells cultured in media without yeast extract were induced at 6 h after inoculation and continued culture for additional 21 h and 45 h. Therefore, the total cultivation time was the same which were 27 h and 51 h, respectively. % P(3HB) content, P(3HB) (mg/L/h), P(3HB) (g/L), and pH of the culture media were determined (Fig 6.21). By using glycerol as a carbon source, cells produced more P(3HB) when compared to glucose. The productivity of P(3HB) was higher when cells were grown in the media without yeast extract. When the time was increased to 51 h, % P(3HB) content and P(3HB) (g/L) were slightly increased but P(3HB) productivity (mg/L/h) was decreased.





E. coli-ABC_{*Ah*} was cultured in three different media which were M9+glucose+yeast extract, M9+glycerol+yeast extract, and M9+glycerol. The cells were induced with 1 mM IPTG at the early log phase. The cells were harvested at 27 h and 51 h after inoculation to evaluate the P(3HB) content, P(3HB) (mg/L/h), P(3HB) (g/L), and pH. Result is the average value and error bar represents standard deviation for triplicate experiments.

6.2.1.4 Effect of stage of cell at the induction point on P(3HB) biosynthesis

The stage of cell was investigated whether it influences the productivity of P(3HB). *E. coli*-ABC_{*Ah*} was cultivated in minimum medium containing glycerol as a carbon source. IPTG was added into the culture medium to induce the expression of PhaABC at four different stages of cell: initial inoculation, early log phase, mid log phase, and late log phase. After induction, cells were cultivated for further 24 h and then harvested and the amount of P(3HB) were analyzed by HPLC (Fig 6.22). When induction at early log phase (3 h after inoculation), % P(3HB) content, P(3HB) (mg/L), and the productivity (mg/L/h) were 23 wt% of CDW, 0.4 mg/L, and 13.1 mg/L/h, respectively. These values were the highest when compared to induction at the other cell stages. The induction at the initial and mid log phase gave similar P(3HB) production. The ability for P(3HB) production was the lowest when the cells were induced at late log phase.





E. coli-ABC_{*Ah*} was cultivated in minimum medium containing glycerol as a sole carbon source. 1 mM IPTG was added into the culture medium at four different stages of cell [at initial inoculation (0 h), early log phase (3 h), mid log phase (7 h), and late log phase (11 h)]. After induction cells were cultivated for further 24 h. The amount of P(3HB) were analyzed by HPLC. Result is the average value and error bar represents standard deviation for triplicate experiments.

6.2.2 SCL-MCL-PHAs biosynthesis

Next, the ability of recombinant *E. coli* strains to synthesize SCL-MCL-PHAs was examined. By cross combinational co-expression of PhaAB with each of three PhaCs and three PhaJs, nine recombinant *E. coli* strains were generated: *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, *E. coli*-ABC2_{*Pp*}J1_{*Pp*}, *E. coli*-ABC2_{*Pp*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J_{*Ah*}.

6.2.2.1 Effect of different PhaC and PhaJ types on SCL-MCL-PHAs biosynthesis

The ability of recombinant strains to convert glycerol to SCL-MCL-PHAs was evaluated by culturing in 300 mL M9 medium containing 2% (v/v) glycerol as a carbon substrate. IPTG (1mM) and sodium dodecanoate (1 g/L) were added to the culture media at 3 h after inoculation. The protein expression profiles were determined for *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, and E. coli-ABC2_{Pp}J1_{Pp} (Fig 6.23), for E. coli-ABC_{Re}J4_{Pp}, E. coli-ABC_{Ah}J4_{Pp}, and E. coli-ABC2_{Pp}J4_{Pp} (Fig 6.24), and for E. coli-ABC_{Re}J_{Ah}, E. coli-ABC_{Ah}J_{Ah}, and E. coli-ABC2_{Pp}J_{Ah} (Fig 6.25). The contents and the compositions of produced copolymers were determined by HPLC. The method for preparation of samples for HPLC analysis was different between P(3HB) and SCL-MCL-PHA copolymer analysis. For analysis of accumulated P(3HB) content, dried cells were digested with H₂SO₄ (138) but this method cannot be used to analyze the compositions of SCL-MCL-PHA copolymer. For analysis of SCL-MCL-PHA copolymer compositions, dried cells were digested with NaOH (139). Figure 6.26 showed the comparison result of HPLC peaks. When P(3HB-6.9 mol% 3HHx) (Nodax) was digested with H₂SO₄, only C4 peak appeared. However, when P(3HB-6.9 mol% 3HHx) was digested with NaOH, both C4 and C6 peaks were detected.



Figure 6.23 Protein expression profiles of *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J1_{*Pp*} as analyzed by SDS-PAGE.

SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J1_{*Pp*} cultured in M9 medium containing 2% (v/v) glycerol supplemented with yeast extract and sodium dodecanoate (1 g/L). The cells were induced with 1 mM IPTG after 3 h inoculation and harvested after 24 h. Lane M is Unstained Protein molecular weight marker. Lanes 1-2, 3-4, 5-6, and 7-8 are the proteins from *E. coli*-ABC_{*Re*}J1_{*Pp*}, *E. coli*-ABC_{*Ah*}J1_{*Pp*}, *E. coli*-ABC2_{*Pp*}J1_{*Pp*}, and *E. coli*-pETDuet-1+pCDFDuet-1, respectively. The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*}, and PhaJ1_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 19 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀. Chitwadee Phithakrotchanakoon

Results / 86



Figure 6.24 Protein expression profiles of *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J4_{*Pp*} as analyzed by SDS-PAGE.

SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, and *E. coli*-ABC2_{*Pp*}J4_{*Pp*} cultured in M9 medium containing 2% (v/v) glycerol supplemented with yeast extract and sodium dodecanoate (1 g/L). The cells were induced with 1 mM IPTG after 3 h inoculation and harvested after 24 h. Lane M is Unstained Protein molecular weight marker. Lanes 1-2, 3-4, 5-6, and 7-8 are the proteins from *E. coli*-ABC_{*Re*}J4_{*Pp*}, *E. coli*-ABC_{*Ah*}J4_{*Pp*}, *E. coli*-ABC2_{*Pp*}J4_{*Pp*}, and *E. coli*-pETDuet-1+pCDFDuet-1, respectively. The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*}, and PhaJ4_{*Pp*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 18 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.



Figure 6.25 Protein expression profiles of *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, and *E. coli*-ABC_{*Pp*}J_{*Ah*} as analyzed by SDS-PAGE.

SDS-PAGE analysis of protein profiles of *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, and *E. coli*-ABC2_{*Pp*}J_{*Ah*} cultured in M9 medium containing 2% (v/v) glycerol supplemented with yeast extract and sodium dodecanoate (1 g/L). The cells were induced with 1 mM IPTG after 3 h inoculation and harvested after 24 h. Lane M is Unstained Protein molecular weight marker. Lanes 1-2, 3-4, 5-6, and 7 are the proteins from *E. coli*-ABC_{*Re*}J_{*Ah*}, *E. coli*-ABC_{*Ah*}J_{*Ah*}, *E. coli*-ABC2_{*Pp*}J_{*Ah*}, and *E. coli*-pETDuet-1+pCDFDuet-1, respectively. The expected size of PhaA_{*Re*}, PhaB_{*Re*}, PhaC_{*Re*}, PhaC_{*Ah*}, PhaC2_{*Pp*}, and PhaJ_{*Ah*} are 42 kDa, 27 kDa, 66 kDa, 68 kDa, 64 kDa, and 16 kDa, respectively. Arrows indicate the expressed recombinant proteins. The samples were loaded at equivalent OD₆₀₀.



Figure 6.26 HPLC analysis of P(3HB-6.9 mol% 3HHx).

The polymers were digested with (A) H_2SO_4 or (B) 1N NaOH. C4 stands for 3HB-derived peak (crotonic acid). C6 stands for 3HHx-derived peak (hexenoic acid). The samples were analyzed by high performance liquid chromatography (HPLC) on an Ultimate 3000 standard LC system (Dionex, Sunnyvale, CA) equipped with a UV detector and an Aminex HPX-87H column (300 mm x 7.8 mm I.D) (Bio-Rad, Hercules, CA) operated at 55°C at a flow rate of 0.5 mL/min. The mobile phase was 20% CH₃CN in 5 mM H₂SO₄ and chromatogram signals were detected at 210 nm.

HPLC analysis showed that *E. coli* expressing PhaABCs were able to produce SCL-MCL-PHA copolymers but the % mole fractions of the MCL-units were very low (less than 1 mol%). Whereas co-expression of PhaABCs and three different PhaJs resulted in the biosynthesis of various SCL-MCL-PHA copolymers consisting of monomers varying from C4 to C10 with various fraction of MCL-units up to 64 mol% (Table 6.3).

Next, some factors that may influence the molar fraction of MCL-unit and % content of produced copolymers were investigated since the molar fraction of MCL-unit in the polymer influences the properties of polymer. These included time-dependent change and concentration of dodecanoate. The candidate strain was *E. coli*-ABC_{*Ah*}J_{*Ah*} which produced P(3HB-*co*-3HHx) copolymer at the highest content with highest 3HHx molar fraction.

with permission from JSBA].					
Studing	PHA content	PHA monomer composition (mol%)			
Strains	(wt% of CDW)	3HB	3HHx	ЗНО	3HD
E. $coli$ -ABC _{Ah}	1.1 ± 0.37	99.4 ± 0.05	0.6 ± 0.05	-	-
E. $coli$ -ABC _{Re}	$0.5 \hspace{0.1 cm} \pm \hspace{0.1 cm} 0.09$	99.1 ± 0.1	$0.85\ \pm 0.1$	0.05 ± 0.02	-
E. $coli$ -ABC2 _{Pp}	trace	nd	nd	nd	nd
E. coli-ABC _{Ah} J1 _{Pp}	$1.0\ \pm 0.17$	$96\ \pm 0.4$	$4\ \pm 0.4$	-	-
E. coli-ABC _{Ah} J4 _{Pp}	1.7 ± 0.49	$98\ \pm 0.3$	2 ± 0.3	-	-
E. coli-ABC _{Ah} J _{Ah}	$1.7 \hspace{0.1in} \pm 0.66$	96 ± 1.5	4 ± 1.5	-	-
E. coli-ABC _{Re} J1 _{Pp}	3.2 ± 0.96	99.6 ± 0.1	0.4 ± 0.1	-	-
E. coli-ABC _{Re} J4 _{Pp}	1.0 ± 0.21	96 ± 0.3	2 ± 0.4	2 ± 0.3	-
$E. coli-ABC_{Re}J_{Ah}$	$0.8\ \pm 0.37$	$96\ \pm 0.6$	3 ± 0.8	1 ± 0.4	-

Table 6.3 PHA copolymers biosynthesis from glycerol and dodecanoate in recombinent E achi berbaring nha APCa and nha APCa la (reproduced from (10))

 65 ± 7.5

 36 ± 4.6

 52 ± 4.9 7 ± 3.2

 6 ± 1.9

 25 ± 1.6

 30 ± 1.5 11 ± 2.1

 13 ± 3.3

 9 ± 3.9

 16 ± 5.8

 30 ± 1.0

 0.1 ± 0.02

 0.1 ± 0.01

 $0.1\ \pm 0.01$

E. coli-ABC2_{*Pp*}J1_{*Pp*}

E. coli-ABC2_{*Pp*}J4_{*Pp*}

E. coli-ABC2_{*Pp*} J_{Ah}

Cells were cultivated at 37°C in M9 minimum medium containing 2% (v/v) glycerol as the sole carbon source supplemented with 1 g/L Bacto-yeast extract. After 3 h of cultivation, IPTG (1mM), sodium dodecanoate (1 g/L) and Brij-35 (2 g/L) were added and cells were grown for an additional 24 h. Results are the average ± standard deviations from four replications. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; CDW, cell dry weight; -, not detectable; nd, not determined.

6.2.2.2 Effect of cultivation time on the P(3HB-co-3HHx) biosynthesis by *E. coli*-ABC_{Ah}J_{Ah}

PHA polymers are produced and accumulated inside the bacterial cells as insoluble granules; therefore the time-dependent change of PHA biosynthesis was investigated. *E. coli*-ABC_{*Ah*}J_{*Ah*} was cultivated in 75 mL M9 minimum medium containing 2% (v/v) glycerol with yeast extract. Sodium dodecanoate (1 g/L) was added into the culture media together with 1mM IPTG after 3 h inoculation. The cells were collected at intervals to measure pH, OD₆₀₀, % PHA content and mol% 3HHx (Fig 6.27).The results showed that after 1 mM IPTG and dodecanoate (1 g/L) were added into the culture media, P(3HB-*co*-3HHx) was gradually accumulated and reached the maximum content at 3.5 wt% of CDW after cultivation for 34 h. The 3HHx fraction reached the maximum at 3 mol% after cultivation for 27 h and then decreased to 1.4 mol% after 34 h and was stable there up to 56 h. pH was decreased from 6.8 at 27 h to 6.1 at 34 h when cells entered into the stationary phase as indicated by OD₆₀₀.


Figure 6.27 Time-dependent change in OD₆₀₀, pH, % PHA content, and mol% 3HHx.

E. coli-ABC_{*Ah*}J_{*Ah*} was cultivated at 37°C in M9 minimum medium containing 2% (v/v) glycerol as the sole carbon source supplemented with Bacto-yeast extract (1 g/L). After 3 h of cultivation, IPTG (1mM), sodium dodecanoate (1 g/L) and Brij-35 (2 g/L) were added. Cells were harvested at interval time to measure OD₆₀₀, pH, % PHA content, and compositions. Results are the average \pm standard deviations from three independent experiments.

6.2.2.3 Effect of dodecanoate concentration on the P(3HB-co-3HHx) biosynthesis by *E. coli*-ABC_{Ah}J_{Ah}

Based on our established hybrid biosynthesis pathway, fatty acid was supplied as the precursor of MCL-monomer. The intermediate of fatty acid β -oxidation was converted to MCL-monomer by the activity of PhaJ. Therefore the amount of dodecanoate (fatty acid) was varied to evaluate its effect on the mol% 3HHx (MCL-unit). *E. coli*-ABC_{*Ah*}J_{*Ah*} was cultured in M9 minimum medium containing glycerol as a carbon source supplemented with yeast extract. After cultivation for 3 h, sodium dodecanoate was added into the culture media at 0.5, 1.0, and 1.5 g/L together with 1mM IPTG. They were cultured for additional 24 h then harvested. PHA content and its compositions were analyzed by HPLC (Table 6.4). The results revealed that cell dry weight, % PHA content, and its compositions were affected by the amount of sodium dodecanoate. When sodium dodecanoate concentration was increased, cell dry weight and % PHA content were decreased; whereas, the molar fraction of 3HHx was increased. The molar fraction of 3HHx was increased from 2 mol% to 5 mol% when sodium dodecanoate was increased from 0.5 g/L to 1.5 g/L.

Media	CDW (g/L)	PHA content (wt% of CDW) —	PHA monomer composition (mol%)	
			3HB	3HHx
А	0.6 ± 0.18	4.6 ± 2.9	98 ± 0.8	2 ± 0.8
В	0.5 ± 0.11	3.4 ± 1.1	97 ± 0.5	3 ± 0.5
С	0.4 ± 0.23	1.5 ± 0.4	95 ± 1.2	5 ± 1.2

Table 6.4 Effect of sodium dodecanoate concentration on PHA production.

E. coli-ABC_{*Ah*}J_{*Ah*} was cultivated at 37°C in M9 minimum medium containing 2% (v/v) glycerol as the sole carbon source supplemented with Bacto-yeast extract (1 g/L). After 3 h of cultivation, IPTG (1mM), sodium dodecanoate [(A) 0.5 g/L, (B) 1 g/L, (C) 1.5 g/L] and Brij-35 (2 g/L) were added and cells were grown for an additional 24 h. Results are the average \pm standard deviations from four replications. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxybexanoate.

6.3 Using crude glycerol as a carbon source for PHAs biosynthesis

A surplus of glycerol generated as by-product from the growing biodiesel industry is a promising inexpensive carbon source (18). Since the high cost of PHAs is their major drawback which limits their used in daily-use plastic products. Thus it is interesting to determine if such crude glycerol can be used as a carbon source to reduce the production cost of PHAs. Two recombinant strains equipped with *phaABC* and *phaABCJ* were used in this experiment. The first strain was *E. coli*-ABC_{*Ah*} which produced P(3HB) homopolymer from pure glycerol at the highest content than the other strains. The second strain was *E. coli*-ABC_{*Ah*} which produced P(3HB-*co*-3HHx) copolymer from pure glycerol at the highest content with highest 3HHx molar fraction.

6.3.1 Characterization of crude glycerol compositions

Crude glycerol in this study was obtained from a local biodiesel production plant which was the by-product of alkaline (NaOH)-catalyzed transesterification of palm oil with methanol (Trang Palm Oil Co. Ltd., Trang, Thailand). The crude glycerol was brown in color (Fig 6.28A). Its viscousity was similar to pure glycerol. After adding crude glycerol into M9 minimum medium, the medium turned to white (Fig 6.28B) which was different from clear M9 minimum medium with pure glycerol. Crude glycerol, as expected, composed of glycerol as a major component [81.1% (w/w)] with impurities of methanol [0.98% and 0.36% (v/v) of before and after autoclaving at 121°C for 15 min, respectively], sodium [0.01% (w/w)], potassium [1.9% (w/w)] and trace amounts of monoglyceride [0.03% (w/w)], diglyceride [0.02% (w/w)], and triglyceride [0.07% (w/w)] (Table 6.5). The possibility of using this crude glycerol as a carbon source for culturing E. coli-ABC_{Ah} and E. coli-ABC_{Ah}J_{Ah} recombinant strains was investigated. E. coli-ABC_{Ah} and E. coli-ABC_{Ah}J_{Ah} were cultivated in shake flask using 1, 3, and 5% (v/v) crude glycerol as a sole carbon source to examine the effect of crude glycerol concentration on their growth (Fig 6.29). Both strains were able to grow using only crude glycerol as a carbon source. When concentration of crude glycerol was increased from 1 to 5% (v/v), there was no significant effect on cell dry weight (CDW) of E. coli-ABC_{Ah}

Fac. of Grad. Studies, Mahidol Univ.

but there was a slight effect on CDW of *E. coli*-ABC_{*Ah*}J_{*Ah*} after 24 h cultivation, with a slight decrease in CDW at higher glycerol concentration.



(A) (

(B)

Figure 6.28 The characteristic of crude glycerol produced from biodiesel production as a by-product.

(A) Crude glycerol from Trang Palm Oil Co. Ltd., Trang, Thailand.(B) M9 minimum medium containing 2% (v/v) crude glycerol.

Parameter	Crude glycerol	Unit
рН	8.9	
Glycerol	81.09	% (w/w)
Methanol (after auto clave)	0.36	% (v/v)
Monoglyceride	0.03	% (w/w)
Diglyceride	0.02	% (w/w)
Triglyceride	0.07	% (w/w)
Sodium	0.01	% (w/w)
Potassium	1.9	% (w/w)

Table 6.5 Compositions of crude glycerol.

(A)





(A) E. coli-ABC_{Ah} and (B) E. coli-ABC_{Ah}J_{Ah} were cultivated in shaking flask at 37°C, 200 rpm using 1%, 3%, and 5% (v/v) crude glycerol as the carbon source. The cultured cells were collected at the interval time to measure the OD_{600} . Result is the average value and error bar represents standard deviation for triplicate experiment.

Fac. of Grad. Studies, Mahidol Univ.

6.3.2 P(3HB) biosynthesis by *E. coli*-ABC_{Ah}

Next, the ability of *E. coli*-ABC_{Ah} to use crude glycerol as a carbon source for biosynthesis of P(3HB) was investigated. E. coli-ABCAh was cultivated in 75 mL M9 medium containing 1% (v/v) of crude glycerol or 1% (v/v) of pure glycerol as the carbon source. When crude glycerol was used, E. coli-ABC_{Ah} produced P(3HB) content at 14 wt% of CDW which was less than when 1% of pure glycerol was used (26 wt% of CDW). However, the total CDW of E. coli cells cultured in crude glycerol was 4.0 g/L which was higher than that of pure glycerol (1.5 g/L). Therefore, the concentration of P(3HB) produced from crude glycerol (0.6 g/L) was higher than that of pure glycerol (0.4 g/L). In addition, the effect of crude glycerol concentration on the ability to synthesize P(3HB) by E. coli-ABC_{Ah} was then investigated. Cells were cultured in 75 mL M9 medium containing 1% to 5% (v/v) of crude glycerol. The results showed that the maximum P(3HB) obtained was 13.9 wt% of CDW by using 1% (v/v) crude glycerol and gradually decreased to 1.6 wt% of CDW by using 5% (v/v) crude glycerol (Table 6.6). The decrease in P(3HB) content was in accordance with the expression level of recombinant proteins (Fig 6.30). When crude glycerol concentration was gradually increased, the expression level of PhaC and PhaA proteins was gradually decreased as evaluated by densitometer (Fig 6.31A and 6.31B, respectively).

Crude glycerol (% v/v)	pH of media	CDW (g/L)	P(3HB) content (wt% of CDW)	P(3HB) (g/L)
1% (pure)	7.0	1.5 ± 0.25	25.5 ± 2.44	0.4 ± 0.07
1 %	7.1	4.0 ± 0.44	13.9 ± 2.52	0.6 ± 0.16
2 %	7.2	3.3 ± 0.17	9.6 ± 0.64	0.3 ± 0.07
3 %	7.3	3.2 ± 0.38	6.4 ± 0.66	0.2 ± 0.03
4 %	7.4	2.7 ± 0.32	3.8 ± 0.42	0.1 ± 0.01
5 %	7.5	2.8 ± 0.13	1.6 ± 0.10	0.05 ± 0.00

Table 6.6 The effect of crude glycerol concentration on P(3HB) biosynthesis in $E. coli-ABC_{Ah}$ [modified from (145) with permission from Springer].

Cells were cultivated at 37°C in 75 mL M9 minimum medium containing glycerol as the sole carbon source supplemented with Bacto-yeast extract (1 g/L). After 3 h of cultivation, IPTG (1mM) was added and cells were grown for an additional 24 h. Results are the average \pm standard deviations from four replications. CDW, cell dry weight.



Figure 6.30 SDS-PAGE analysis of protein expression levels in *E. coli*-ABC_{Ah} cultured in crude glycerol [reproduced from (145) with permission from Springer].

The recombinant cells were cultured in 75 mL M9 medium with different % of crude glycerol. The lanes indicated as 0 h represents expression profile at the induction time point (3 h after inculation). The lanes indicated as 24 h show the expression profile at 24 h after induction by 1 mM IPTG. The calculated sizes of PhaC_{*Ah*}, PhaA_{*Re*}, and PhaB_{*Re*} are 68, 42, and 27 kDa, respectively and their expected migrations are indicated on the right. Arrows indicate the expected positions of recombinant proteins. The samples were loaded at equivalent cultured volume.

	۸
14	1
· · ·	

(B)



Figure 6.31 Densitometry analysis of protein band intensity.

Protein intensity level of $PhaC_{Ah}$ (A) and $PhaA_{Re}$ (B) expressed by *E. coli*-ABC_{Ah} cultured in different crude glycerol concentration as a sole carbon source were analyzed by SynGene GeneTools (version: 4.03.02.0). The calculated relative intensity values of recombinant proteins are shown in the table. Fac. of Grad. Studies, Mahidol Univ.

6.3.3 P(3HB-co-3HHx) biosynthesis by E. coli-ABC_{Ah}J_{Ah}

To investigate the conversion of crude glycerol to P(3HB-*co*-3HHx), *E. coli*-ABC_{*Ah*}J_{*Ah*} was cultured in 75 mL M9 medium containing 1% (v/v) crude glycerol as a sole carbon source. HPLC analysis revealed that by using crude glycerol as a sole carbon source, *E. coli*-ABC_{*Ah*}J_{*Ah*} produced P(3HB) homopolymer (Table 6.7). Therefore, to provide precursors for MCL-monomer synthesis, fatty acid was added into the culture media. Here, sodium dodecanoate (1 g/L) was added at the induction time together with 1 mM IPTG and further incubated for 24 h. Cell dry weight, % PHA content, and PHA compositions were evaluated (Table 6.7). HPLC analysis showed that *E. coli*-ABC_{*Ah*}J_{*Ah*} produced P(3HB-*co*-HHx) at 3 wt% of CDW with 1 mol% 3HHx fraction from crude glycerol which is less than 4 wt% of CDW with 3 mol% 3HHx fraction produced from pure glycerol. However, the total CDW of *E. coli* cells cultured in crude glycerol was 2.8 g/L which was higher than that of pure glycerol (0.9 g/L). Therefore, the concentration of produced P(3HB-*co*-3HHx) (g/L) from crude glycerol was higher than that of pure glycerol.

Table 6.7 PHA biosynthesis in *E. coli*-ABC_{*Ah*}J_{*Ah*} using pure glycerol (PG) and crude glycerol (CG) with or without sodium dodecanoate (C12) [modified from (145) with permission from Springer].

Media	CDW PHA conte (g/L) (wt% of CD	PHA content	PHA monomer composition (mol%)	
		(wt/% of CDW)	3HB	3HHx
PG	1.6 ± 0.09	15.9 ± 0.31	100	-
CG	2.4 ± 0.13	6.6 ± 0.77	100	-
PG + C12	0.9 ± 0.16	4.3 ± 1.50	97 ± 1.5	3 ± 1.5
CG + C12	2.8 ± 0.59	2.9 ± 1.92	99 ± 0.3	1 ± 0.3

Cells were cultivated at 37°C in 75 mL M9 minimum medium containing 1 % (v/v) pure or crude glycerol as a carbon source supplemented with Bacto-yeast extract (1 g/L). After 3 h of cultivation, IPTG (1 mM) with or without sodium dodecanoate (1 g/L) were added and cells were grown for an additional 24 h. Results are the average \pm standard deviations from three independent experiments. CDW, cell dry weight; -, not detectable.

6.4 PHA properties characterization

To investigate the effect of incorporated MCL-unit on their polymer properties, some PHAs produced [SCL-PHA: P(3HB) homopolymer produced from pure glycerol by *E. coli*-ABC_{*Re*}; and SCL-MCL-PHAs: P(3HB-1 mol% 3HHx) and P(3HB-3 mol% 3HHx) produced from crude and pure glycerol by *E. coli*-ABC_{*Ah*}J_{*Ah*}, respectively] were further characterized.

6.4.1 Chemical structure analysis by NMR

The extracted polymer polymers were dried under vacuum prior to characterize by ¹H NMR and ¹³C NMR analysis. The NMR spectra of P(3HB-3 mol% 3HHx) copolymer was compared to that of P(3HB) homopolymer. P(3HB) exhibited three groups of chemical shifts (δ) in ¹H NMR at 1.23 ppm [3HB (3)], 2.45-2.60 ppm [3HB (1)], and 5.23 ppm [3HB (2)] for the methyl groups (-CH₃), the methylene groups (-CH₂), and the methine groups (-CH), respectively (Fig 6.32A). Whereas P(3HB-3 mol% 3HHx) exhibited the same spectral patterns as described above but with additional δ values of 0.83 ppm and 1.55 ppm, which represented the other positions of the terminal-CH₃ [3HHx (5)] and the internal-CH₂ [3HHx (3)], respectively. (Fig 6.32B). These results suggested that this polymer composed not only 3HB but also MCL-unit in the polymer chains. In addition, the ¹H NMR spectra intensity ratio of the main chain methylene proton resonance [3HB (1), 3HHx (1)] to methyl proton resonance [3HHx (5)] of copolymers were calculated to obtain the molar fractions of 3HB and 3HHx unit (Equation 1). The result corresponded to the HPLC analysis which indicated that 3 mol% 3HHx was incorporated into the polymer. Moreover. ¹³C NMR spectra of P(3HB-3 mol% 3HHx) were consistent with the previously characterized P(3HB-co-3HHx) (146). The expanded spectra of carbonyl resonance between 169.2-169.7 ppm split to four peaks resulted from the different sequential connection of 3HB and 3HHx units (Fig 6.33B), in contrast to the only one peak of P(3HB) (Fig 6.33A).

Fac. of Grad. Studies, Mahidol Univ.

Equation 1

% molar fraction of 3HHx =
$$\left[\frac{\text{intensity of 3HHx (5) peak / 3}}{(\text{intensity of 3HB (1) + 3HHx (1)) / 2}}\right] \times 100$$

= $\left[\frac{0.09 / 3}{(1.01 + 1.03) / 2}\right] \times 100 = 2.94\%$
(A)







¹H NMR spectra of P(3HB) extracted from *E. coli*-ABC_{*Re*} (A) compared to P(3HB-3 mol% 3HHx) copolymers extracted from *E. coli*-ABC_{*Ah*}J_{*Ah*} (B).





¹³C NMR spectra of P(3HB) extracted from *E. coli*-ABC_{*Re*} (A) compared to P(3HB-3 mol% 3HHx) copolymers extracted from *E. coli*-ABC_{*Ah*}J_{*Ah*} (B). a*a, refers to 3HB connected to 3HB; a*b and b*a, refer to 3HB connected to 3HHx and vice versa; b*b, refers to 3HHx connected to 3HHx.

6.4.2 Molecular weight analysis by GPC

The molecular weight and the polydispersity index of PHAs produced were determined by GPC (Table 6.8). The average molecular weights (M_w) of P(3HB), P(3HB-1 mol% 3HHx), and P(3HB-3 mol% 3HHx) were in range of (1.1-2.6) x10⁵ Da. Their polydispersity index which indicates their heterogeneousity with higher value, denoting greater heterogeneousity were around 2.0.

6.4.3 Thermal properties analysis by DSC

The thermal properties including glass transition temperature (T_g) and melting temperature (T_m) of P(3HB), P(3HB-1 mol% 3HHx), and P(3HB-3 mol% 3HHx) were determined by DSC (Table 6.8). T_g is the temperature at which the polymer is converted between glassy and rubbery states while T_m is the temperature at which the polymer is converted between rubbery and melting states. T_g values of P(3HB), P(3HB-1 mol% 3HHx), and P(3HB-3 mol% 3HHx) were 7.5°C, 6.9°C, and 5.5°C, respectively. DSC thermogram of P(3HB) showed only one T_m peak at 175.0°C (Fig 6.34A), while DSC thermogram of P(3HB-1 mol% 3HHx) showed one distinct T_m peak with a shoulder at 174.3°C (Fig 6.34B). On the other hand, DSC thermograms of P(3HB-3 mol% 3HHx) showed two T_m peaks at 158.7°C and 171.3°C. These observed two peaks might be resulted from the heterogeneity of produced copolymer, the multiple crystalline forms, or due to the recrystallization during the heating runs in the DSC where the lower T_m peak is the real melting point of original crystals and the higher T_m peak is the melting point of crystal formed by recrystallization (147).

6.4.4 Degradation temperature analysis by TGA

P(3HB), P(3HB-1 mol% 3HHx) and P(3HB-3 mol% 3HHx) were characterized by thermogravimetry analysis (TGA) to evaluate their thermal degradation temperature (T_d). T_d is the temperature at which the polymer is started to loss of its physical, mechanical or electrical properties. The results showed that T_d value of P(3HB) homopolymer was 291.7°C which was slightly lower than those of P(3HB-1 mol% 3HHx) and P(3HB-3 mol% 3HHx) copolymers (299.4°C and 294.6°C, respectively) (Table 6.8).

	Molecular weight		Ther	Thermal properties (°C)		
Type of PHAs	$M_{w}(x10^{5})$	PDI	$T_{ m g}$	$T_{ m m}$	T _d	
P(3HB)	2.6	1.9	7.5	175.0	291.7	
P(3HB-1 mol% 3HHx)	1.1	2.1	6.9	174.3	299.4	
P(3HB-3 mol% 3HHx)	1.3	2.0	5.5	158.7, 171.3	294.6	

Table 6.8 Molecular weight and thermal properties of PHAs [modified from (145)with permission from Springer].

 M_w , weight average molecular weight; PDI, polydispersity index; T_g , glass transition temperature; T_m , melting temperature; T_d , degradation temperature.



Figure 6.34 DSC thermogram of PHAs.

P(3HB) (A), P(3HB-*co*-3HHx) extracted from *E. coli*-ABC_{Ah}J_{Ah} cultured in pure (B) and crude glycerol (C). T_g , glass transition temperature; T_m , melting temperature.

CHAPTER VII DISCUSSION

7.1 Establishment of PHA biosynthesis pathway in recombinant *E. coli*

The compositions of PHAs produced are dependent on three major factors which are the feeding substrate, the metabolic route for providing monomers and the activity of PHA synthase (38, 39). Since the compositions of PHAs influence the properties of polymer materials, the challenge is to produce controllable compositions of PHAs. In this thesis, the hybrid pathway which combines two monomer supplying routes mimicking from two groups of native PHA-producing bacteria and one polymerase enzyme which can polymerize both SCL-monomer and MCL-monomer was designed (Fig 1.2). The advantage of the hybrid pathway is that the fraction between SCL-unit and MCL-unit in SCL-MCL-PHA copolymer can be easily controlled by adjusting the concentration ratio of glycerol and fatty acid. This similar strategy was also used to control the fraction of 3HP and 4HB in P(3HP-co-4HB) E. coli by using 1,3-propanediol and 1,4-butanediol as co-substrates produced from (148). Other strategies can also control the ratio between SCL and MCL but are more complicated. For example, the fractions of 3HP and 3HB are controlled under different inducible promoters of their monomer supplying routes in recombinant E. coli for biosynthesis of P(3HP-co-3HB) from glycerol (149). The lactic acid fraction (LA) in P(LA-co-3HB) was regulated by site-specific mutagenesis of the polymerase enzyme (150). Moreover, in our study three different polymerase enzymes (PhaCs) and three different MCL-monomer supplying related enzymes (PhaJs) were varied. Therefore, a variation of compositions of produced PHA would be expected.

Two sets of recombinant plasmids were constructed which were pETDuet-*ABC*s and pCDFDuet-*J*s. First, *phaABC*s were cloned into the same vector since these three genes encode the enzymes for P(3HB) biosynthesis pathway. Next, *phaJ*s were cloned into pCDFDuet-1 vector and co-expressed with PhaABCs to

complete the pathway for biosynthesis of SCL-MCL-PHA copolymers. Recombinant *E. coli* strains could express all recombinant proteins (PhaABCsJs) as indicated by SDS-PAGE and western blot analysis. The expression level of three different PhaCs was different. It was found that $PhaC2_{Pp}$ was expressed at the lowest level. However, in cell expressing $PhaC2_{Pp}$, the expression level of PhaA was higher than that of the other two strains, probably because they share the common resource pool for protein biosynthesis.

7.2 Using pure glycerol as a carbon source for PHAs biosynthesis

In this study, E. coli was selected as a host strain for PHAs production due to several advantages such as the ability to utilize several carbon substrates, fast growth to high cell density, and lack of intracellular PHA depolymerase (78, 79). Native E. coli is unable to produce and accumulate PHA granules. Therefore, some strategies of metabolic engineering approaches which were heterologous gene expression and substrate precursor addition were applied to establish the bio-system for PHA biosynthesis in E. coli (81). PHA synthase (PhaC) which is the key enzyme for polymerization of the (R)-3-hydroxyacyl-CoA monomers to PHAs were varied. Different PhaC enzymes from three sources showed effect on the ability to produce P(3HB). Nile blue A fluorescence staining analysis suggested that E. coli-ABC_{Re} and E. coli-ABC_{Ah} accumulated PHA granules inside the cells (Fig 6.16). However, HPLC results revealed that all three strains: E. coli-ABC_{Re}, E. coli-ABC_{Ah}, and *E.* coli-ABC2_{Pp} could produce and accumulate P(3HB) (Table 6.1). These differences between Nile blue A staining and HPLC analysis results may be due to the sensitivity limitation of Nile blue A staining. Under the cultured condition used, E. coli-ABC_{Ah} accumulated P(3HB) as similar to E. coli-ABC_{Re} at 30 wt% and 24 wt%, respectively. On the other hand, E. coli-ABC2_{Pp} accumulated P(3HB) only at 2 wt% of CDW. Since PhaC2_{Pp} was originated from P. putida KT2440 which was reported as a MCL-PHA producer (151), PhaC2_{Pp} tends to prefer MCL-3HA-CoA monomers than (R)-3HB-CoA as substrate. This preference may cause low content of P(3HB)produced from *E. coli*-ABC2_{*Pp*}. Another possibility may be due to the low expression level of PhaC2_{Pp} (Fig 6.15) as there is a report that the expression level of PhaC

correlates to the amount of P(3HB) produced (152). However, based on our results, the PhaC expression level and the amount of accumulated P(3HB) were correlated only when the same PhaC was used. Since PhaC_{Ah} was expressed at lower level than PhaC_{Re} but *E. coli*-ABC_{Ah} produced more P(3HB) than *E. coli*-ABC_{Re} (Fig 6.15). The results suggested that besides the expression level, the types of PhaCs which would have different specific activity may affect P(3HB) production.

Since E. coli-ABC_{Ah} gave highest P(3HB) content from glycerol, it was further used as a producer to investigate other cultivation parameters. The results suggested that the ability to synthesize P(3HB) by E. coli-ABC_{Ah} was not significantly affected by increasing the concentration of pure glycerol. This is because CDW, % P(3HB) content, and P(3HB) concentration were only slightly decreased at 1.2, 1.2, and 1.4 fold, respectively, with increasing glycerol concentration. The amount of glycerol remaining after 27 h cultivation indicated that the amount of glycerol used was in excess. On the other hand, when a common-use carbon source like glucose was used, less amount of P(3HB) was obtained than that obtained from glycerol used at the same concentration (g/L) and in excess amount. This might result from different carbon catabolism of glycerol and glucose. Since glycerol has lower oxidation state than glucose, its catabolism generates more reducing equivalents (NADH) and leads to the higher ratio of NADH/NAD+ (153). In order to maintain the redox balance, the cells have to drive carbon flow toward the synthesis of reduced products including P(3HB). Moreover, it is known that NADH significantly inhibits citrate synthase activity, thus causing more available acetyl-CoA molecules for P(3HB) biosynthesis (154). On the other hand, the pH of culture media after 27 h cultivation with glucose was lower than that of glycerol suggesting that, apart from P(3HB), other acids might be produced in the culture media with glucose. The addition of yeast extract into the culture media supported better growth of the cells but the amount of P(3HB) produced is decreased. This is not surprising as it is known that most of native PHA-producing strains accumulate PHA when growing in the nitrogen-limited media. These results suggested that P(3HB) biosynthesis in recombinant strain might be accelerated by high carbon/nitrogen ratio. Furthermore, it was suspected that the stage of cells at the induction time would influence the production of P(3HB). In fact, most of the investigations induce protein expression at early log phase (87, 155, 156) but some of them induce at the initial (14, 157) or using a two-stage fed-batch culture dividing a cell growth phase and a P(3HB) accumulation phase (158). As expected, the P(3HB) productivity was highest when induced at early log phase of cells and less P(3HB) was found if induced at late log phase.

Also, the MCL monomers-supplying route was established by co-transformation of pCDFDuet-Js together with pETDuet-ABCs into E. coli. The biosynthesis of SCL-MCL-PHA copolymers from glycerol was investigated. E. coli-ABC_{Ah}, E. coli-ABC_{Re}, and E. coli-ABC_{2p} were used as control strains. HPLC analysis revealed that co-expression of only PhaABC (without PhaJ) also produced SCL-MCL-PHA copolymers when dodecanoate was supplemented into the culture media (Table 6.3). This suggested that E. coli BL21(DE3) possesses some endogenous enzymes or some metabolic routes that can provide MCL-monomers for PHA synthase. Previous reports revealed that some enzymes of E. coli can catalyze the intermediates of fatty acid β-oxidation to MCL-3HA-CoA monomer molecules. For example, 3-ketoacyl-ACP reductase (FabG) from E. coli catalyzes 3-ketoacyl-CoA to (R)-3-HA-CoA (91) and MaoC form E. coli has PhaJ-like function by catalyzing 2-enoyl-CoA to (R)-3-HA-CoA (94). However, based on our result, only a small fraction of MCL-units (less than 1 mol%) were incorporated in the polymers, suggesting that the activity level of its endogenous enzyme was very low since this heterologous pathway has to compete with the inherent pathways for intermediates. Therefore, PhaJ enzymes were co-expressed with PhaABCs in recombinant E. coli to increase the fraction of MCL-units.

HPLC analysis indicated that co-expressing PhaA and PhaB with various PhaCs and PhaJs in recombinant *E. coli* (*E. coli*-ABCsJs) caused the strains to produce various SCL-MCL-PHA copolymers (Table 6.3). However, the % content values of PHA copolymers were not high (0.1-3.2 wt% of cell dry weight). By comparing the amount of PHA produced by *E. coli*-ABC_{Ah} in the medium with or without dodecanoate, it was found that P(3HB) was accumulated at 22 wt% of CDW when 2% glycerol was used as a sole carbon source (Table 6.2), whereas when sodium dodecanoate was supplemented into the culture media % PHA content was dramatically decreased to only 1 wt% of CDW. This reduction might be due to the toxicity effect of fatty acid (dodecanoate) on the cell. The action of fatty acids on

bacterial cell still unclear, but the prime target seems to be the membrane and on various processes occurring at the membrane of bacteria (159, 160). Therefore one possible way to optimize the productivity is by using fed-batch cultivation approach to gradually feeding fatty acid into the culture media.

Considering the compositions of PHA produced, PhaC may be the most important factor in determining copolymer compositions. PHAs composing of 3HB (C4) and 3HHx (C6) were obtained from cell expressing $PhaC_{Ah}$ suggesting that PhaC_{Ah} prefers to C4 and C6 monomers. This was not unexpected as it was reported that PhaC_{Ac} prefers to C4 and C6 monomers (13, 95). Besides 3HB (C4) and 3HHx (C6), the longer monomer 3HO (C8) was detected in cells expressing $PhaC_{Re}$, this suggested that $PhaC_{Re}$ has broader substrate specificity than $PhaC_{Ah}$. Although native R. eutropha is reported to produce P(3HB) (140) and the in vitro study indicated that substrate specificity of $PhaC_{Re}$ is toward only SCL-monomers (161), our study revealed that in recombinant host equipped with the pathway providing both SCLmonomer and MCL-monomer, not only SCL-monomer but also MCL-monomer could be polymerized by PhaC_{Re}. While in cells expressing PhaC_{Ah} or PhaC_{Re} produced copolymers composing mainly 3HB-unit with a small fraction of MCL-unit (up to 4 mol%), cells expressing PhaC2_{Pp} produced copolymers composing at least half of MCL-unit fraction (48-64 mol%) and longer monomer 3HD (C10) was also detected. This is the first study demonstrating the *in vivo* substrate specificity of PhaC2_{*Pp*}. The result suggested that $PhaC2_{Pp}$ could polymerize both SCL-monomer and MCLmonomers and has broader substrate specificity than $PhaC_{Ah}$ and $PhaC_{Re}$.

On the other hand, considering the effect of PhaJ on the compositions of PHAs produced, the result indicated that PhaJs provided various monomers up to ten carbon chain length (C10). Also, this study revealed, for the first time, the *in vivo* substrate specificity of PhaJ_{Ah}. Although native *A. hydrophila* ATCC 7966 produces PHA composed of 3HB (C4) and 3HHx (C6) (31), the substrate specificity of PhaJ_{Ah} was not limited to only C4 and C6 but also the longer monomers. In addition, the PHA compositions in cells expressing PhaC2_{Pp} (*E. coli*-ABC2_{Pp}J1_{Pp}, *E. coli*-ABC2_{Pp}J4_{Pp}, and *E. coli*-ABC2_{Pp}J_{Ah}) showed that the dominant monomers 3HO (C8) and 3HD (C10) were detected when cells expressed PhaJ1_{Pp} and PhaJ4_{Pp}, whereas the shorter monomer 3HHx (C6) and 3HO (C8) were detected in cells expressing PhaJ_{Ah}. These

suggested that various PhaJ variants influenced the dominant MCL-monomer in the PHA produced. Therefore, the variable compositions of SCL-MCL-PHAs could be produced by matching different monomer supplying enzymes (PhaJs) with different polymerase enzymes (PhaCs).

Based on these results, one candidate strain, *E. coli*-ABC_{*Ah*}J_{*Ah*} was selected to investigate its biosynthesis of P(3HB-*co*-3HHx) over time (56 h). The results indicated that the content and compositions of P(3HB-*co*-3HHx) in *E. coli*-ABC_{*Ah*}J_{*Ah*} is time-dependent. After induction, the polymer content was gradually increased and reached the maximum when cell enter into the stationary phase. The fraction of 3HHx was sharply increased to the maximum at 3 mol% in the exponential growth phase. However, later, the 3HHx fraction was decreased to 1.4 mol%. This might be due to the less active β -oxidation in cell decreasing growth rate, the loss of recombinant plasmid, or the lesser amount of remaining dodecanoate after longer cultivation. It was then confirmed that the fraction of 3HHx was correlated to the amount of dodecanoate (Table 6.4). These suggested that, the fraction of MCL-units could be regulated by the amounts of fatty acid precursor substrate.

Taken together, these data demonstrated that the constructed recombinant *E. coli* strains are able to produce P(3HB) and SCL-MCL-PHAs by using pure glycerol as a carbon source.

7.3 Using crude glycerol as a carbon source for PHA biosynthesis

The compositions of crude glycerol depend on the starting materials, the method of transesterification, and the recovery process (109). Since crude glycerol used in this study was partially purified; it was quite pure, consisting of 81% glycerol with smaller fractions of impurities (methanol, salts, and mono-, di-, and triglycerides); this is in contrast to some other previously characterized crude glycerol sources which contained 63-77% glycerol and 23-38% methanol (162). The possibility of utilizing this crude glycerol as a carbon source for biosynthesis of PHAs by our constructed recombinant *E. coli* strains was investigated. The result indicated that *E. coli*-ABC_{Ah} was able to grow using only crude glycerol as a carbon source. Moreover, the total cell dry weight of *E. coli* cells cultured in 1% (v/v) crude glycerol

was higher than that in pure glycerol. This suggested that some impurities in crude glycerol such as mono-, di-, triglyceride might enhance the bacterial growth. Teeka et al., (2010) also found that an unidentified bacteria strain, AIK7, grows better in crude glycerol (163). However, some previous studies found that some impurities especially sodium salt in crude glycerol suppressed the growth of bacteria (2, 130). Since our crude glycerol contained small amount of sodium salt ~0.01% (w/w), this might not affect cell growth. However, when the concentration of crude glycerol was gradually increased from 1% to 5% (v/v), the concentration of P(3HB) was gradually decreased from 14 wt% to 2 wt% of CDW, respectively. Furthermore, the expression level of recombinant proteins was also decreased with increasing crude glycerol concentration. These suggested that in the recombinant strains, crude glycerol concentration affected the expression level of recombinant proteins which then directly influence the amount of accumulated P(3HB). On the other hand, the decrease in protein expression level and P(3HB) productivity could have resulted from the effect of higher osmotic stress on the cells and/or the effect of some conditions such as higher pH and salts that might impede recombinant proteins expression and polymer biosynthesis (164). It should be noted that when the concentration of pure glycerol was increased from 1% to 5% (v/v), the % P(3HB) content was only slightly decreased (1.2) fold); whereas, when the concentration of crude glycerol was increased from 1% to 5% (v/v), the % P(3HB) content was significantly decreased (8.7 fold).

Based on this result, 1% (v/v) crude glycerol was then tested for its possibility to use as a carbon source for biosynthesis of P(3HB-*co*-3HHx) copolymer by *E. coli*-ABC_{*Ah*}J_{*Ah*}. When crude glycerol was used, *E. coli*-ABC_{*Ah*}J_{*Ah*} only produced P(3HB) homopolymer, indicating that using just crude glycerol cannot lead to the production of SCL-MCL-PHAs. This is in contrast to the previous study which demonstrated that *Pseudomonas corrugata* can use crude glycerol as a sole carbon source for biosynthesis of MCL-PHA suggesting that fatty acid precursors remaining in crude glycerol undergoes β -oxidation to provide MCL-monomers (132). It should be noted that crude glycerol used in that contains fatty acid soap, fatty acid methyl ester, mono-, and di-glyceride in total at 34% which were approximately 300 times higher than that present in the crude glycerol used in our study (mono-, di-, tri-glycerides in total at 0.12%). Therefore, it is possible that the fatty acid precursors

remaining in our crude glycerol might not be enough to be converted to MCL-monomers for copolymer biosynthesis. By adding dodecanoate into the culture media, recombinant *E. coli*-ABC_{*Ah*}J_{*Ah*} is able to produce P(3HB-*co*-HHx) copolymer as expected. The results suggest that crude glycerol used in this study has a potential to use as a carbon source for recombinant cells growth and PHAs biosynthesis.

7.4 PHA properties characterization

The weight average molecular weight (M_w) values of P(3HB), P(3HB-1 mol% 3HHx), and P(3HB-3 mol% 3HHx) produced in this study were in the degree of 10^5 Da suggesting that the recombinant strains produced high molecular weight polymers comparable to the previous studies using glycerol for PHA biosynthesis (122, 131, 165). However these obtained values were slightly lower than those obtained from copolymers produced by recombinant *E. coli* using glucose as a carbon source [(4.6-6.5) x 10^5 Da] (166). According to the previous studies, it has been demonstrated that lower M_w of PHAs (122, 124, 131) was because glycerol can act as an end-cap to terminate the polymerization reaction (167).

On the other hand, incorporation of 3HHx fraction into the polymer causes changes in the thermal properties of produced PHAs. The results showed that glass transition temperature (T_g) values were inversely correlated to the 3HHx fraction (Table 6.8), in which increasing 3HHx fraction decreased the values of T_g . In general, the lower T_g values are resulted from the higher of the segmental mobility of polymer chains (147, 168). Therefore, the incorporation of 3HHx unit, which contains longer flexible side-chain than 3HB, resulted in copolymers with higher segmental mobility. In addition, the incorporation of 3HHx unit also affected the melting temperature (T_m) of polymers in which increasing 3HHx fraction resulted in decreasing T_m of P(3HB*co*-3HHx) copolymers. This result suggested that the crystallinity of P(3HB) homopolymer was decreased by 3HHx incorporation. Since typical PHAs suffer from thermal degradation during the melting process (168), increasing the gap between melting temperature and thermal decomposition temperature is required. Therefore the lower T_m of P(3HB-*co*-3HHx) copolymer is advantageous because the material melting process can be carried out at lower temperature than its thermal decomposition condition.

Moreover, the results of TGA analysis showed that a small fraction of 3HHx incorporated in the polymers slightly increased their thermal degradation temperature (T_d) , up to 299°C. During thermal degradation, P(3HB) undergoes a random chain scission via β -hydrogen transfer through the formation of a sixmembered ring intermediate (169). Therefore, the steric hindrance of propyl side chain of 3HHx might disturb the formation of six-membered ring which causes the increase in the thermal degradation temperature of P(3HB-co-3HHx) when compared to P(3HB). However, the difference was not significant as the fraction of 3HHx was not high. Besides the MCL-unit fraction that affects the thermal stability of polymers, the presence of some impurities, such as some fermentation residues or some salts in polymers, may also affect the thermal stability (169, 170). Considering the T_d values of P(3HB), P(3HB-1 mol% 3HHx), and P(3HB-3 mol% 3HHx) extracted from the recombinant strains in this study, the results indicated that T_d of these PHAs are superior than those of previously obtained from P(3HB) produced from glycerol by Burkholderia cepacia (281.5°C) (124) or from P(3HB-co-3HHx) produced from wheat germ oil by A. hydrophila 7966 (~250°C) (31). Taken together, the results suggested that the PHA polymers produced from either pure or crude glycerol by our recombinant E. coli possess the properties suitable for various industrial applications such as softeners, pharmaceutical agents, and also in some nano-particles (130, 171, 172).

CHAPTER VIII CONCLUSIONS

In this study, the recombinants E. coli with ability to convert glycerol to PHAs have been successfully developed. The recombinant E. coli strains harboring β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) from R. eutropha and a PHA synthase enzyme (PhaC) from either R. eutropha, A. hydrophila, or P. putida showed the ability to utilize glycerol as a sole carbon source for biosynthesis of P(3HB). The difference in the expression level and the specific activity/substrate specificity of the three PhaCs influence the amount of accumulated P(3HB). Under the same cultivation conditions, cells expressing PhaAB with PhaC from R. eutropha (E. coli-ABC_{Re}) or PhaC from A. hydrophila (E. coli-ABC_{Ah}) produced similar amount of P(3HB); whereas, cells expressing PhaAB with PhaC2 from P. puida (E. coli-ABC2_{Pp}) produced the lowest amount of P(3HB). The cultivation parameters including types of carbon source, carbon: nitrogen ratio, and the stage of the cells at the induction point are found to be important for further optimization of P(3HB) production. Using glucose as a carbon source, E. coli-ABC_{Ah} produced less amount of P(3HB) than using glycerol; while, high carbon: nitrogen ratio and the induction at the early exponential growth phase accelerated the ability to synthesize P(3HB) from glycerol by *E. coli*-ABC_{*Ah*}.

Furthermore, the hybrid pathway for biosynthesis of SCL-MCL-PHA copolymers has been established. Co-expressing PhaAB with three different PhaCs and three different PhaJs led to the production of various types of SCL-MCL-PHA copolymers. The copolymers produced consisted of C4 to C10 monomers with various fractions of MCL-units up to 64 mol%. In this study, *in vivo* substrate specificities of two PhaC enzymes from *A. hydrophila* ATCC 7966 (PhaC_{Ah}) and *P. putida* KT2440 (PhaC2_{Pp}) were identified, i.e. PhaC_{Ah} is specified to C4 and C6 monomers, while PhaC2_{Pp} had broader substrate specificity toward C4 to C10 monomers. Moreover, the results demonstrated that PhaJ from *A. hydrophila* (PhaJ_{Ah}) could produce various

types of MCL-monomer, up to C10. Also, the correlation between the molar fraction of MCL-unit and the amount of dodecanoate was demonstrated which was increasing amount of dodecanoate increased molar fraction of MCL-unit.

This work also showed that crude glycerol can be used as a sole carbon source for biosynthesis of P(3HB). *E. coli*-ABC_{*Ah*} produced P(3HB) at 14 wt% of CDW from 1% (v/v) crude glycerol. *E. coli*-ABC_{*Ah*}J_{*Ah*} produced P(3HB-*co*-3HHx) copolymer by using crude glycerol supplemented with dodecanoate. Although both recombinant strains (*E. coli*-ABC_{*Ah*} and *E. coli*-ABC_{*Ah*}J_{*Ah*}) accumulated less % content of PHAs from crude glycerol than pure glycerol, the higher amount of total CDW was obtained from crude glycerol, thus leading to higher PHA concentration (g/L).

Incorporation of 3HHx unit (MCL-unit) in polymer decreased both glass transition temperature (T_g) and melting temperature (T_m) of P(3HB) homopolymer. These properties are superior to P(3HB) homopolymer and are required for ease of melting process.

In my thesis work, I have constructed several recombinant *E. coli* strains for production of PHAs from glycerol and provided information for optimization of higher PHA production yield from glycerol with the designed properties to achieve overall improvement in economic competitiveness in the bio-plastic industry. Moreover, this work will encourage the development of PHA biosynthesis system integrated with biodiesel production to reduce the production cost of PHA and simultaneously reduces the problem of surplus crude glycerol.

REFERENCES

- Castilho, L. R., Mitchell, D. A., Freire, D. M.: Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation, Bioresour Technol, 100, 5996-6009 (2009).
- 2.Cavalheiro, J. M. B. T., de Almeida, M. C. M. D., Grandfils, C., da Fonseca, M. M. R.: Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol, Process Biochem, 44, 509-515 (2009).
- 3.Chee, J. Y., Tan, Y., Samian, M. R., Sudesh, K.: Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols, J Polym Environ, 18, 584-592 (2010).
- 4.Sudesh, K., Abe, H., Doi, Y.: Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters, Prog Polym Sci, 25, 1503-1555 (2000).
- 5.Harding, K. G., Dennis, J. S., von Blottnitz, H., Harrison, S. T.: Environmental analysis of plastic production processes: comparing petroleum-based polypropylene and polyethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis, J Biotechnol, **130**, 57-66 (2007).
- 6.Jung, Y. K., Lee, S. Y., Tam, T. T.: Towards systems metabolic engineering of PHA producers, plastic from bacteria, 14, 63-84 (2010).
- 7.Matsusaki, H., Abe, H., Doi, Y.: Biosynthesis and properties of poly(3hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant strains of *Pseudomonas sp. 61-3*, Biomacromolecules, 1, 17-22 (2000).
- 8.Abe, H., Doi, Y., Fukushima, T., Eya, H.: Biosynthesis from gluconate of a random copolyester consisting of 3-hydroxybutyrate and medium-chainlength 3-hydroxyalkanoates by *Pseudomonas* sp. 61-3, Int J Biol Macromol, 16, 115-119 (1994).

- 9.Doi, Y., Kitamura, S., Abe, H.: Microbial synthesis and characterization of poly(3hydroxybutyrate-co-3-hydroxyhexanoate), Macromolecules, 28, 4822-4828 (1995).
- 10.Lee, S. H., Oh, D. H., Ahn, W. S., Lee, Y., Choi, J., Lee, S. Y.: Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by high-cell-density cultivation of *Aeromonas hydrophila*, Biotechnol Bioeng, 67, 240-244 (2000).
- 11.Janda, J. M., Abbott, S. L.: The genus *Aeromonas*: taxonomy, pathogenicity, and infection, Clin Microbiol Rev, 23, 35-73 (2010).
- 12.Tsuge, T.: Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates, J Biosci Bioeng, 94, 579-584 (2002).
- 13.Fukui, T., Doi, Y.: Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxybexanoate) biosynthesis genes of *Aeromonas caviae*, J Bacteriol, 179, 4821-4830 (1997).
- 14.Antonio, R. V., Steinbuchel, A., Rehm, B. H.: Analysis of in vivo substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyesters in recombinant *Escherichia coli*, FEMS Microbiol Lett, 182, 111-117 (2000).
- 15.Nomura, C. T., Taguchi, K., Taguchi, S., Doi, Y.: Coexpression of genetically engineered 3-ketoacyl-ACP synthase III (fabH) and polyhydroxyalkanoate synthase (phaC) genes leads to short-chain-length-medium-chain-length polyhydroxyalkanoate copolymer production from glucose in *Escherichia coli* JM109, Appl Environ Microbiol, **70**, 999-1007 (2004).
- 16.Zou, X. H., Chen, G. Q.: Metabolic engineering for microbial production and applications of copolyesters consisting of 3-hydroxybutyrate and mediumchain-length 3-hydroxyalkanoates, Macromol Biosci, 7, 174-182 (2007).
- 17.Choi, J., Lee, S. Y.: Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation, Bioprocess Eng 17, 335-342 (1997).

- 18.Yazdani, S. S., Gonzalez, R.: Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr Opin Biotechnol, 18, 213-219 (2007).
- 19.Phithakrotchanakoon, C., Champreda, V., Aiba, S., Pootanakit, K., Tanapongpipat, S.: Engineered *Escherichia coli* for Short-Chain-Length Medium-Chain-Length polyhydroxyalkanoate copolymer biosynthesis from glycerol and dodecanoate, Biosci Biotechnol Biochem, 77, 1262-1268 (2013).
- 20.Lemoigne, M.: Products of dehydration and of polymerization of 3-hydroxybutyric acid, Bull Soc Chim Biol, 8, 770-782 (1926).
- 21.Zhu, C., Chiu, S., Nakas, J. P., Nomura, T. N.: Bioplastics from waste glycerol derived from biodiesel industry, J Appl Polym Sci, 130, 1-13 (2013).
- 22.Dawes, E. A., Senior, P. J.: The role and regulation of energy reserve polymers in microorganisms, Adv Microb Physiol, 10, 135-266 (1973).
- 23.Findlay, R. H., White, D. C.: Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*, Appl Environ Microbiol, 45, 71-78 (1983).
- 24. Wallen, L. L., Rohwedder, W. K.: Poly-beta-hydroxyalkanoate from activated sludge, Environ Sci Technol, 8, 576-579 (1974).
- 25.de Smet, M. J., Eggink, G., Witholt, B., Kingma, J., Wynberg, H.: Characterization of intracellular inclusions formed by Pseudomonas oleovorans during growth on octane, J Bacteriol, **154**, 870-878 (1983).
- 26.Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G., Witholt, B.: Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates, Appl Environ Microbiol, 54, 2924-2932 (1988).
- 27.Slater, S. C., Voige, W. H., Dennis, D. E.: Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-beta-hydroxybutyrate biosynthetic pathway, J Bacteriol, **170**, 4431-4436 (1988).

- 28.Schubert, P., Steinbuchel, A., Schlegel, H. G.: Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*, J Bacteriol, **170**, 5837-5847 (1988).
- 29.**Rehm, B. H.:** Polyester synthases: natural catalysts for plastics, Biochem J, **376**, 15-33 (2003).
- 30.Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D., Radecka, I.: Bacterial synthesis of biodegradable polyhydroxyalkanoates, J Appl Microbiol, 102, 1437-1449 (2007).
- 31.Asrar, J., Valentin, H. E., Berger, P. A., Tran, M., Padgette, S. R., Garbow, J.
 R.: Biosynthesis and properties of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) polymers, Biomacromolecules, 3, 1006-1012 (2002).
- 32.Wang, Y. W., Yang, F., Wu, Q., Cheng, Y. C., Yu, P. H. F., Chen, J., Chen, G.
 Q.: Effect of composition of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) on growth of fibroblast and osteoblast, Biomaterials, 26, 755-761 (2005).
- 33.Madison, L. L., Huisman, G. W.: Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic, Microbiol Mol Biol Rev, 63, 21-53 (1999).
- 34.Rehm, B. H.: Bacterial polymers: biosynthesis, modifications and applications, Nat Rev Microbiol, 8, 578-592 (2010).
- 35.Potter, M., Madkour, M. H., Mayer, F., Steinbuchel, A.: Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16, Microbiology, 148, 2413-2426 (2002).
- 36.Dawes, E. A., Senior, P. J.: The role and regulation of energy reserve polymers in micro-organisms, Adv Microb Physiol, 10, 135-266 (1973).
- 37.Jendrossek, D., Handrick, R.: Microbial degradation of polyhydroxyalkanoates, Annu Rev Microbiol, 56, 403-432 (2002).
- 38.Anderson, A. J., Dawes, E. A.: Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates, Microbiol Rev, 54, 450-472 (1990).
- 39.Steinbüchel, A., Valentin, H. E.: Diversity of bacterial polyhydroxyalkanoic acids, FEMS Microbiol Lett, 128, 219-228 (1995).

- 40.Kessler, B., Witholt, B.: Factors involved in the regulatory network of polyhydroxyalkanoate metabolism, J Biotechnol, **86**, 97-104 (2001).
- 41.Park, S. J., Choi, J., Lee, S. Y.: Engineering of *Escherichia coli* fatty acid metabolism for the production of polyhydroxyalkanoates, Enzyme Microb Tech, 36, 579-588 (2005).
- 42.Lennen, R. M., Pfleger, B. F.: Engineering *Escherichia coli* to synthesize free fatty acids, Trends Biotechnol, **30**, 659-667 (2012).
- 43.**Rehm, B. H., Kruger, N., Steinbuchel, A.:** A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. The PHAG gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase, J Biol Chem, **273**, 24044-24051 (1998).
- 44.Valentin, H. E., Steinbuchel, A.: Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene, Appl Microbiol Biotechnol, **39**, 309-317 (1993).
- 45.**Tombolini, R., Povolo, S., Buson, A., Squartini, A., Nuti, M. P.:** Poly-betahydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41, Microbiology, **141**, 2553-2559 (1995).
- 46.Ramachander, T. V., Rawal, S. K.: PHB synthase from *Streptomyces aureofaciens* NRRL 2209, FEMS Microbiol Lett, **242**, 13-18 (2005).
- 47.**Khanna, S., Srivastava, A. K.:** Recent advances in microbial polyhydroxyalkanoates, Process Biochem, **40**, 607-619 (2005).
- 48.**Reinecke, F., Steinbüchel, A.:** *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers, J Mol Microb Biotech, **16**, 91-108 (2009).
- 49.Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Potter, M., Schwartz, E. *et al*: Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16, Nat Biotechnol, 24, 1257-1262 (2006).
- 50.Luengo, J. M., García, B., Sandoval, A., Naharro, G., Olivera, E. R.: Bioplastics from microorganisms, Curr Opin Microbiol, 6, 251-260 (2003).

- 51.Kim, B. S., Chul Lee, S. C., Lee, S. Y., Chang, H. N., Chang, Y. K., Woo, S. L.: Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control, Biotechnol Bioeng, 43, 892-898 (1994).
- 52.Doi, Y., Segawa, A., Nakamura, S., Kunioka, M.: Production of biodegradable copolyesters by *Alcaligenes eutrophus*, Novel biodegradable microbial polymers, 186, 37-48 (1990).
- 53.Du, G. C., Chen, J., Yu, J., Lun, S.: Feeding strategy of propionic acid for production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with *Ralstonia eutropha*, Biochem Eng J, 8, 103-110 (2001).
- 54.Green, P. R., Kemper, J., Schechtman, L., Guo, L., Satkowski, M., Fiedler, S., Steinbuchel, A., Rehm, B. H.: Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid betaoxidation inhibited *Ralstonia eutropha*, Biomacromolecules, **3**, 208-213 (2002).
- 55.Silva-Queiroz, S. R., Silva, L. F., Pradella, J. G. C., Pereira, E. M., Gomez, J. G. C.: PHA(MCL) biosynthesis systems in *Pseudomonas aeruginosa* and *Pseudomonas putida* strains show differences on monomer specificities, J Biotechnol, 143, 111-118 (2009).
- 56.Huijberts, G. N. M., Eggink, G.: Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures, Appl Microbiol Biotechnol, 46, 233-239 (1996).
- 57.Ramsay, B. A., Saracovan, I., Ramsay, J. A., Marchessault, R. H.: Continuous production of long-side-chain poly-beta-hydroxyalkanoates by *Pseudomonas oleovorans*, Appl Environ Microbiol, 57, 625-629 (1991).
- 58.Haywood, G. W., Anderson, A. J., Ewing, D. F., Dawes, E. A.: Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135, Appl Environ Microbiol, 56, 3354-3359 (1991).
- 59.**Timm, A., Steinbüchel, A.:** Formation of polyesters consisting of medium-chainlength 3-hydroxyalkanoic acids from gluconate by *Pseudomonas*

aeruginosa and other fluorescent *pseudomonads*, Appl Environ Microbiol, **56**, 3360-3367 (1990).

- 60. Witholt, B., Kessler, B.: Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics, Curr Opin Biotechnol, 10, 279-285 (1999).
- 61.**Timmis, K. N.:** *Pseudomonas putida*: a cosmopolitan opportunist par excellence, Environ Microbiol, **4**, 779-781 (2002).
- 62.Wang, Q., Nomura, C. T.: Monitoring differences in gene expression levels and polyhydroxyalkanoate (PHA) production in *Pseudomonas putida* KT2440 grown on different carbon sources, J Biosci Bioeng, **110**, 653-659 (2010).
- 63.Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., dos Santos,
 V. A. P. M., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M. *et al*: Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440, Environ Microbiol, 4, 799-808 (2002).
- 64.Huisman, G. W., Wonink, E., Meima, R., Kazemier, B., Terpstra, P., Witholt,
 B.: Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas* oleovorans. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA, J Biol Chem, 266, 2191-2198 (1991).
- 65.Timm, A., Steinbuchel, A.: Cloning and molecular analysis of the poly(3hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1, Eur J Biochem, 209, 15-30 (1992).
- 66.Matsusaki, H., Manji, S., Taguchi, K., Kato, M., Fukui, T., Doi, Y.: Cloning and molecular analysis of the Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) biosynthesis genes in *Pseudomonas* sp. strain 61-3, J Bacteriol, 180, 6459-6467 (1998).
- 67.Kim, T. K., Shin, H. D., Seo, M. C., Lee, J. N., Lee, Y. H.: Molecular structure of PCR cloned PHA synthase genes of *Pseudomonas putida* KT2440 and its utilization for medium-chain length polyhydroxyalkanoate production, J Microbiol Biotechnol, **13**, 182-190 (2003).

- 68.**Tsuge, T., Taguchi, K., Taguchi, S., Doi, Y.:** Molecular characterization and properties of (R)-specific enoyl-CoA hydratases from *Pseudomonas aeruginosa*: metabolic tools for synthesis of polyhydroxyalkanoates via fatty acid beta-oxidation, Int J Biol Macromol, **31**, 195-205 (2003).
- 69.Sato, S., Kanazawa, H., Tsuge, T.: Expression and characterization of (R)-specific enoyl coenzyme A hydratases making a channeling route to polyhydroxyalkanoate biosynthesis in *Pseudomonas putida*, Appl Microbiol Biotechnol, **90**, 951-959 (2011).
- 70.Seshadri, R., Joseph, S. W., Chopra, A. K., Sha, J., Shaw, J., Graf, J., Haft, D.,
 Wu, M., Ren, Q., Rosovitz, M. J. et al: Genome sequence of Aeromonas hydrophila ATCC 7966T: jack of all trades, J Bacteriol, 188, 8272-8282 (2006).
- 71.Chen, G. Q., Zhang, G., Park, S. J., Lee, S. Y.: Industrial scale production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), Appl Microbiol Biotechnol, 57, 50-55 (2001).
- 72.Lu, X. Y., Wu, Q., Chen, G. Q.: Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) with flexible 3-hydroxyhexanoate content in *Aeromonas hydrophila* CGMCC 0911, Appl Microbiol Biotechnol, 64, 41–45 (2004).
- 73.Kato, M., Bao, H. J., Kang C. K., Fukui, T., Doi, Y.: Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars, Appl Microbiol Biotechnol, 45, 363-370 (1996).
- 74.Steinbüchel, A., Wiese, S.: A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids, Appl Microbiol Biotechnol, 37, 691-697 (1992).
- 75.Liebergesell, M., Hustede, E., Timm, A., Steinbüchel, A., Fuller, R. C., Lenz,
 R. W., Schlegel, H. G.: Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria, Arch Microbiol, 155, 415-421 (1991).
- 76.**Haywood, G. W., Anderson, A. J., Williams, D. R., Dawes, E. A., Ewing, D. F.:** Accumulation of a poly(hydroxyalkanoate) copolymer containing

primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126, Int J Biol Macromol, **13**, 83-88 (1991).

- 77.Fukui, T., Shiomi, N., Doi, Y.: Expression and characterization of (*R*)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*, J Bacteriol, **180**, 667-673 (1998).
- 78.Lee, S. Y.: E. coli moves into the plastic age, Nat Biotechnol, 15, 1997 (1997).
- 79.Lee, S. Y., Choi, J. I.: Production of microbial polyester by fermentation of recombinant microorganisms, Adv Biochem Eng Biotechnol, 71, 183-207 (2001).
- 80.Li, R., Zhang, H., Qi, Q.: The production of polyhydroxyalkanoates in recombinant *Escherichia coli*, Bioresour Technol, **98**, 2313-2320 (2007).
- 81.Aldor, I. S., Keasling, J. D.: Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates, Curr Opin Biotechnol, 14, 475-483 (2003).
- 82.Miyake, M., Miyamoto, C., Schnackenberg, J., Kurane, R., Asada, Y.: Phosphotransacetylase as a key factor in biological production of polyhydroxybutyrate, Appl Biochem Biotechnol, 84-86, 1039-1044 (2000).
- 83.Kabir, M. M., Shimizu, K.: Fermentation characteristics and protein expression patterns in a recombinant *Escherichia coli* mutant lacking phosphoglucose isomerase for poly(3-hydroxybutyrate) production, Appl Microbiol Biotechnol, **62**, 244-255 (2003).
- 84.Jung, Y. M., Lee, J. N., Shin, H. D., Lee, Y. H.: Role of *tktA* gene in pentose phosphate pathway on odd-ball biosynthesis of poly-beta-hydroxybutyrate in transformant *Escherichia coli* harboring phbCAB operon, J Biosci Bioeng, 98, 224-227 (2004).
- 85.Fiedler, S., Steinbuchel, A., Rehm, B. H.: The role of the fatty acid betaoxidation multienzyme complex from *Pseudomonas oleovorans* in polyhydroxyalkanoate biosynthesis: molecular characterization of the *fadBA* operon from *P. oleovorans* and of the enoyl-CoA hydratase genes *phaJ* from *P. oleovorans* and *Pseudomonas putida*, Arch Microbiol, **178**, 149-160 (2002).
- 86.Tsuge, T., Fukui, T., Matsusaki, H., Taguchi, S., Kobayashi, G., Ishizaki, A., Doi, Y.: Molecular cloning of two (R)-specific enoyl-CoA hydratase genes from *Pseudomonas aeruginosa* and their use for polyhydroxyalkanoate synthesis, FEMS Microbiol Lett, **184**, 193-198 (2000).
- 87.Ren, Q., Sierro, N., Witholt, B., Kessler, B.: FabG, an NADPH-dependent
 3-ketoacyl reductase of *Pseudomonas aeruginosa*, provides precursors for medium-chain-length poly-3-hydroxyalkanoate biosynthesis in *Escherichia coli*, J Bacteriol, 182, 2978-2981 (2000).
- 88. Taguchi, K., Aoyagi, Y., Matsusaki, H., Fukui, T., Doi, Y.: Co-expression of 3-ketoacyl-ACP reductase and polyhydroxyalkanoate synthase genes induces PHA production in *Escherichia coli* HB101 strain, FEMS Microbiol Lett, **176**, 183-190 (1999).
- 89.**Hoffmann, N., Steinbüchel, A., Rehm, B. H.:** The *Pseudomonas aeruginosa phaG* gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources, FEMS Microbiol Lett, **184**, 253-259 (2000).
- 90.Ren, Q., Sierro, N., Kellerhals, M., Kessler, B., Witholt, B.: Properties of engineered poly-3-hydroxyalkanoates produced in recombinant *Escherichia coli* strains, Appl Environ Microbiol, 66, 1311-1320 (2000).
- 91.Park, S. J., Park, J. P., Lee, S. Y.: Metabolic engineering of *Escherichia coli* for the production of medium-chain-length polyhydroxyalkanoates rich in specific monomers, FEMS Microbiol Lett, 214, 217-222 (2002).
- 92.Qi, Q., Steinbuchel, A., Rehm, B. H.: Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid beta-oxidation by acrylic acid, FEMS Microbiol Lett, 167, 89-94 (1998).
- 93.Langenbach, S., Rehm, B. H., Steinbuchel, A.: Functional expression of the PHA synthase gene phaC1 from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis, FEMS Microbiol Lett, 150, 303-309 (1997).
- 94.Park, S. J., Lee, S. Y.: Identification and characterization of a new enoyl coenzyme A hydratase involved in biosynthesis of medium-chain-length

polyhydroxyalkanoates in recombinant *Escherichia coli*, J Bacteriol, **185**, 5391-5397 (2003).

- 95.Park, S. J., Ahn, W. S., Green, P. R., Lee, S. Y.: Production of poly(3hydroxybutyrate-co-3-hydroxyhexanoate) by metabolically engineered *Escherichia coli* strains, Biomacromolecules, 2, 248-254 (2001).
- 96.Davis, R., Anilkumar, P. K., Chandrashekar, A., Shamala, T. R.: Biosynthesis of polyhydroxyalkanoates co-polymer in *E. coli* using genes from *Pseudomonas* and *Bacillus*, Anton Leeuw Int J G, 94, 207-216 (2008).
- 97.Tokiwa, Y., Calabia, B. P.: Degradation of microbial polyesters, Biotechnol Lett, 26, 1181-1189 (2004).
- 98.Phithakrotchanakoon, C., Rudeekit, Y., Tanapongpipat, S., Leejakpai, T., Aiba, S., Noda, I., Champreda, V.: Microbial degradation and physicochemical alteration of polyhydroxyalkanoates by a thermophilic *Streptomyces* sp., Biologia, 64, 246-251 (2009).
- 99.**Philip, S., Keshavarz, T., Roy, I.:** Polyhydroxyalkanoates: biodegradable polymers with a range of applications, J Chem Technol Biotechnol, **82**, 233-247 (2007).
- 100.Mergaert, J., Anderson, C., Wouters, A., Swings, J.: Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in compost, J Environ Polym Degr, 2, 177-183 (1994).
- 101.Mergaert, J., Webb, A., Anderson, C., Wouters, A., Swings, J.: Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in soils, Appl Environ Microbiol, 59, 3233-3238 (1993).
- 102.Mergaert, J., Wouters, A., Anderson, C., Swings, J.: In situ biodegradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in natural waters, Can J Microbiol, 41, 154-159 (1995).
- 103.Mukai, K., Doi, Y., Sema, Y., Tomita, K.: Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases, Biotechnol Lett, 15, 601-604 (1993).

- 104.Lobler, M., Sass, M., Michel, P., Hopt, U. T., Kunze, C., Schmitz, K. P.: Differential gene expression after implantation of biomaterials into rat gastrointestine, J Mater Sci Mater Med, 10, 797-799 (1999).
- 105.Volova, T., Shishatskaya, G., Sevastianov, V., Efremov, S., Mogilnaya, O.: Results of biomedical investigations of PHB and PHB/PHV fibers, Biochem Eng J, 16, 125-133 (2003).
- 106.Ye, C., Hu, P., Ma, M. X., Xiang, Y., Liu, R. G., Shang, X. W.: PHB/PHBHHx scaffolds and human adipose-derived stem cells for cartilage tissue engineering, Biomaterials, **30**, 4401-4406 (2009).
- 107.Chen, G. Q.: Plastics completely synthesized by bacteria: polyhydroxyalkanoates, Microbiology Monographs (plastic from bacteria), 14, 17-37 (2010).
- 108. Chen, G. Q., Wu, Q.: The application of polyhydroxyalkanoates as tissue engineering materials, Biomaterials, 26, 6565-6578 (2005).
- 109. Chatzifragkou, A., Papanikolaou, S.: Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes, Appl Microbiol Biotechnol, 95, 13-27 (2012).
- 110.Yang, F., Hanna, M. A., Sun, R.: Value-added uses for crude glycerol--a byproduct of biodiesel production, Biotechnol Biofuels, 5, 13 (2012).
- 111.da Silva, G. P., Mack, M., Contiero, J.: Glycerol: a promising and abundant carbon source for industrial microbiology, Biotechnol Adv, 27, 30-39 (2009).
- 112.Peters, D.: Raw materials, Adv Biochem Eng Biotechnol, 105, 1-30 (2007).
- 113.Murarka, A., Dharmadi, Y., Yazdani, S. S., Gonzalez, R.: Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals, Appl Environ Microbiol, 74, 1124-1135 (2008).
- 114.Johnson, D. T., Taconi, K. A.: The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production, Environ Prog, 26, 338-348 (2007).

- 115.Cerrate, S., Yan, F., Wang, Z., Coto, C., Sacakli, P., Waldroup, P. W.: Evaluation of glycerine from biodiesel production as a feed ingredient for broilers, Int J Poul Sci, 5, 1001-1007 (2006).
- 116.Dasaria, M. A., Kiatsimkula, P., Sutterlinb, W. R., Suppes, G. J.: Lowpressure hydrogenolysis of glycerol to propylene glycol, Appl Catal A: Gen, 281, 225-231 (2005).
- 117.Mattam, A. J., Clomburg, J. M., Gonzalez, R., Yazdani, S. S.: Fermentation of glycerol and production of valuable chemical and biofuel molecules, Biotechnol Lett, 35, 831-842 (2013).
- 118.Ito, T., Nakashimada, Y., Senba, K., Matsui, T., Nishio, N.: Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process, J Biosci Bioeng, 100, 260-265 (2005).
- 119.Kenny, S. T., Runic, J. N., Kaminsky, W., Woods, T., Babu, R. P., O'Connor,
 K. E.: Development of a bioprocess to convert PET derived terephthalic acid and biodiesel derived glycerol to medium chain length polyhydroxyalkanoate, Appl Microbiol Biotechnol, 95, 623-633 (2012).
- 120.Moralejo-Garate, H., Mar'atusalihat, E., Kleerebezem, R., van Loosdrecht, M. C.: Microbial community engineering for biopolymer production from glycerol, Appl Microbiol Biotechnol, 92, 631-639 (2011).
- 121.Kang, Z., Du, L. L., Kang, J. H., Wang, Y., Wang, Q., Liang, Q. F., Qi, Q. S.: Production of succinate and polyhydroxyalkanoate from substrate mixture by metabolically engineered *Escherichia coli*, Bioresour Technol, **102**, 6600-6604 (2011).
- 122.Kawata, Y., Aiba, S.: Poly(3-hydroxybutyrate) production by isolated Halomonas sp. KM-1 using waste glycerol, Biosci Biotechnol Biochem, 74, 175-177 (2010).
- 123.Shrivastav, A., Mishra, S. K., Shethia, B., Pancha, I., Jain, D., Mishra, S.: Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing Jatropha biodiesel byproduct, Int J Biol Macromol, 47, 283-287 (2010).
- 124.**Zhu, C., Nomura, C. T., Perrotta, J. A., Stipanovic, A. J., Nakas, J. P.:** Production and characterization of poly-3-hydroxybutyrate from biodiesel-

glycerol by *Burkholderia cepacia* ATCC 17759, Biotechnol Prog, **26**, 424-430 (2010).

- 125.Andreessen, B., Lange, A. B., Robenek, H., Steinbuchel, A.: Conversion of glycerol to poly(3-Hydroxypropionate) in recombinant *Escherichia coli*, Appl Environ Microbiol, **76**, 622-626 (2010).
- 126.Ibrahim, M. H., Steinbuchel, A.: Zobellella denitrificans strain MW1, a newly isolated bacterium suitable for poly(3-hydroxybutyrate) production from glycerol, J Appl Microbiol, **108**, 214-225 (2010).
- 127.Ibrahim, M. H., Steinbuchel, A.: Poly(3-hydroxybutyrate) production from glycerol by *Zobellella denitrificans* MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction, Appl Environ Microbiol, 75, 6222-6231 (2009).
- 128.Nikel, P. I., Pettinari, M. J., Galvagno, M. A., Mendez, B. S.: Poly(3hydroxybutyrate) synthesis from glycerol by a recombinant *Escherichia coli* arcA mutant in fed-batch microaerobic cultures, Appl Microbiol Biotechnol, **77**, 1337-1343 (2008).
- 129.Almeida, A., Nikel, P. I., Giordano, A. M., Pettinari, M. J.: Effects of granuleassociated protein PhaP on glycerol-dependent growth and polymer production in poly(3-hydroxybutyrate)-producing *Escherichia coli*, Appl Environ Microbiol, **73**, 7912-7916 (2007).
- 130. Mothes, G., Schnorpfeil, C., Ackermann, J.-U.: Production of PHB from crude glycerol, Eng Life Sci, 7, 475-479 (2007).
- 131.Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, P., Kroutil, M., Martinz, J., Neto, J., Pereira, L., Varila, P.: Production of polyhydroxyalkanoates from agricultural waste and surplus materials, Biomacromolecules, 6, 561-565 (2005).
- 132.Ashby, R. D., Solaiman, D. K. Y., A., F. T.: Bacterial Poly(hydroxyalkanoate) Polymer Production from the Biodiesel Co-product Stream, J Polym Environ, 12, 105-112 (2004).
- 133. Mahishi, L. H., Tripathi, G., Rawal, S. K.: Poly(3-hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* harbouring *Streptomyces*

aureofaciens PHB biosynthesis genes: Effect of various carbon and nitrogen sources, Microbiol Res, **158**, 19-27 (2003).

- 134.Aldor, I. S., Kim, S. W., Prather, K. L., Keasling, J. D.: Metabolic engineering of a novel propionate-independent pathway for the production of poly(3hydroxybutyrate-co-3-hydroxyvalerate) in recombinant *Salmonella enterica serovar typhimurium*, Appl Environ Microbiol, **68**, 3848-3854 (2002).
- 135.Bormann, E. J., Roth, M.: The production of polyhydroxybutyrate by *Methylobacterium rhodesianum* and *Ralstonia eutropha* in media containing glycerol and casein hydrolysates, Biotechnol Lett, **21**, 1059-1063 (1999).
- 136.**Sambrook, J., Fritsch, E. F., Maniatis, T.:**Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New york,
- 137.**Ostle, A. G., Holt, J. G.:** Nile blue A as a fluorescent stain for poly-betahydroxybutyrate, Appl Environ Microbiol, **44**, 238-241 (1982).
- 138.Hesselmann, R. P., Fleischmann, T., Hany, R., Zehnder, A. J.: Determination of polyhydroxyalkanoates in activated sludge by ion chromatographic and enzymatic methods, J Microbiol Meth, 35, 111-119 (1999).
- 139.Watanabe, Y., Ichinomiya, Y., Shimada, D., Saika, A., Abe, H., Taguchi, S., Tsuge, T.: Development and validation of an HPLC-based screening method to acquire polyhydroxyalkanoate synthase mutants with altered substrate specificity, J Biosci Bioeng, 113, 286-292 (2012).
- 140.Sangkharak, K., Prasertsan, P.: Utilization of biodiesel waste as a feedstock for the production of polyhydroxybutyrate by *Cupriavidus necator*, Afr J Biotechnol, 10, 17812-17824 (2011).
- 141.Sun, Z. Y., Ramsay, J. A., Guay, M., Ramsay, B. A.: Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440, Appl Microbiol Biotechnol, 71, 423-431 (2006).
- 142.Fukui, T., Yokomizo, S., Kobayashi, G., Doi, Y.: Co-expression of polyhydroxyalkanoate synthase and (*R*)-enoyl-CoA hydratase genes of *Aeromonas caviae* establishes copolyester biosynthesis pathway in *Escherichia coli*, FEMS Microbiol Lett, **170**, 69-75 (1999).

- 143.Sato, S., Ishii, N., Hamada, Y., Abe, H., Tsuge, T.: Utilization of 2-alkenoicacids for biosynthesis of medium-chain-length polyhydroxyalkanoates in metabolically engineered *Escherichia coli* to construct a novel chemical recycling system, Polymer Degradation and Stability, **97**, 329-336 (2012).
- 144.**Hisano, T., Tsuge, T., Fukui, T., Iwata, T., Miki, K., Doi, Y.:** Crystal structure of the (*R*)-specific enoyl-CoA hydratase from *Aeromonas caviae* involved in polyhydroxyalkanoate biosynthesis, J Biol Chem, **278**, 617-624 (2003).
- 145.Phithakrotchanakoon, C., Champreda, V., Aiba, S., Pootanakit, K., Tanapongpipat, S.: Production of polyhydroxyalkanoates from crude glycerol using recombinant *Escherichia coli*, J Polym Environ, *in press* (2014).
- 146.Shimamura, E., Kasuya, K., Kobayashi, G., Shiotani, T., Shima, Y., Doi, Y.: Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), Macromolecules, 27, 878-880 (1994).
- 147.Watanabe, T., He, Y., Fukuchi, T., Inoue, Y.: Comonomer compositional distribution and thermal characteristics of bacterially synthesized poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)s, Macromol Biosci, 1, 75-83 (2001).
- 148.Meng, D. C., Shi, Z. Y., Wu, L. P., Zhou, Q., Wu, Q., Chen, J. C., Chen, G.
 Q.: Production and characterization of poly(3-hydroxypropionate-co-4-hydroxybutyrate) with fully controllable structures by recombinant Escherichia coli containing an engineered pathway, Metab Eng, 14, 317-324 (2012).
- 149.Wang, Q., Yang, P., Xian, M., Yang, Y., Liu, C., Xue, Y., Zhao, G.: Biosynthesis of poly(3-hydroxypropionate-co-3-hydroxybutyrate) with fully controllable structures from glycerol, Bioresour Technol, 142, 741-744 (2013).
- 150. Yamada, M., Matsumoto, K., Shimizu, K., Uramoto, S., Nakai, T., Shozui, F., Taguchi, S.: Adjustable mutations in lactate (LA)-polymerizing enzyme

for the microbial production of LA-based polyesters with tailor-made monomer composition, Biomacromolecules, **11**, 815-819 (2010).

- 151.Sun, Z., Ramsay, J. A., Guay, M., Ramsay, B. A.: Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440, Appl Microbiol Biotechnol, 74, 69-77 (2007).
- 152.Agus, J., Kahar, P., Abe, H., Doi, Y., Tsuge, T.: Altered expression of polyhydroxyalkanoate synthase gene and its effect on poly[(*R*)-3hydroxybutyrate] synthesis in recombinant *Escherichia coli*, Polym Degrad Stabil, **91**, 1645-1650 (2006).
- 153.San, K. Y., Bennett, G. N., Berríos-Rivera, S. J., Vadali, R. V., Yang, Y. T., Horton, E., Rudolph, F. B., Sariyar, B., Blackwood, K.: Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*, Metab Eng, 4, 182-192 (2002).
- 154.**Henderson, R. A., Jones, C. W.:** Poly-3-hydroxybutyrate production by washed cells of *Alcaligenes eutrophus*; purification, characterisation and potential regulatory role of citrate synthase, Arch Microbiol, **168**, 486-492 (1997).
- 155. Valentin, H. E., Mitsky, T. A., Mahadeo, D. A., Tran, M., Gruys, K. J.: Application of a propionyl coenzyme A synthetase for poly(3hydroxypropionate-*co*-3-hydroxybutyrate) accumulation in recombinant *Escherichia coli*, Appl Environ Microbiol, **66**, 5253-5258 (2000).
- 156.Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., Zhao, G.: Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*, Bioresour Technol, **131**, 548-551 (2013).
- 157.Lu, X., Zhang, J., Wu, Q., Chen, G. Q.: Enhanced production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) via manipulating the fatty acid beta-oxidation pathway in *E. coli*, FEMS Microbiol Lett, 221, 97-101 (2003).
- 158.Kahar, P., Agus, J., Kikkawa, Y., Taguchi, K., Doi, Y., Takeharu Tsuge, T.: Effective production and kinetic characterization of ultra-high-molecularweight poly[(R)-3-hydroxybutyrate] in recombinant *Escherichia coli*, Polym Degrad Stabil, 87, 161-169 (2005).

- 159.Desbois, A. P., Smith, V. J.: Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential, Appl Microbiol Biotechnol, 85, 1629-1642 (2010).
- 160.Sprong, R. C., Hulstein, M. F., Van der Meer, R.: Bactericidal activities of milk lipids, Antimicrob Agents Chemother, 45, 1298-1301 (2001).
- 161.**Haywood, G. W., Anderson, A. J., Dawes, E. D.:** The importance of PHBsynthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*, FEMS Microbiol Lett, **57**, 1-6 (1989).
- 162.**Thompson, J. C., He, B. B.:** Characterization of crude glycerol from biodiesel production from multiple feedstocks, Appl Eng Agric, **22**, 261-265 (2006).
- 163.Teeka, J., Imai, T., Cheng, X., Reungsang, A., Higuchi, T., Yamamoto, K., Sekine, M.: Screening of PHA-producing bacteria using biodiesel-derived waste glycerol as a sole carbon source, J Water Environ Tech, 8, 373-381 (2010).
- 164.Lee, S. J., Kim, S. B., Kang, S. W., Han, S. O., Park, C., Kim, S. W.: Effect of crude glycerol-derived inhibitors on ethanol production by *Enterobacter* aerogenes, Bioprocess Biosyst Eng, 35, 85-92 (2012).
- 165.Teeka, J., Imai, T., Reungsang, A., Cheng, X., Yuliani, E., Thiantanankul, J., Poomipuk, N., Yamaguchi, J., Jeenanong, A., Higuchi, T. *et al*: Characterization of polyhydroxyalkanoates (PHAs) biosynthesis by isolated *Novosphingobium* sp. THA_AIK7 using crude glycerol, J Ind Microbiol Biotechnol, **39**, 749-758 (2012).
- 166.Nomura, C. T., Tanaka, T., Gan, Z. H., Kuwabara, K., Abe, H., Takase, K., Taguchi, K., Doi, Y.: Effective enhancement of short-chain-lengthmedium-chain-length polyhydroxyalkanoate copolymer production by coexpression of genetically engineered 3-ketoacyl-acyl-carrier-protein synthase III (fabH) and polyhydroxyalkanoate synthesis genes, Biomacromolecules, 5, 1457-1464 (2004).
- 167.Madden, L. A., Anderson, A. J., Shah, D. T., Asrar, J.: Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxycompounds as chain transfer agents, Int J Biol Macromol, 25, 43-53 (1999).

- 168.Noda, I., Green, P. R., Satkowski, M. M., Schechtman, L. A.: Preparation and properties of a novel class of polyhydroxyalkanoate copolymers, Biomacromolecules, 6, 580-586 (2005).
- 169.Hablot, E., Bordes, P., Pollet, E., Ave´rous, L.: Thermal and thermo-mechanical degradation of poly(3-hydroxybutyrate)-based multiphase systems, Polym Degrad Stabil, 93, 413-421 (2008).
- 170.Kopinke, F. D., Remmler, M., Mackenzie, K.: Thermal decomposition of biodegradable polyesters—I: Poly(β-hydroxybutyric acid), Polym Degrad Stabil, 52, 25–38 (1996).
- 171.**Foster, L. J. R.:** PEGylation and BioPEGylation of polyhydroxyalkanotes: synthesis, characterisation and applications, Biopolymers, 243-256 (2010).
- 172.**Chen, G. Q.:** A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry, Chem Soc Rev, **38**, 2434-2446 (2009).

Ph.D.(Molecular Genetics and Genetic Engineering) / 137

BIOGRAPHY

NAME	Miss Chitwadee Phithakrotchanakoon
DATE OF BIRTH	27 September 1982
PLACE OF BIRTH	Chonburi, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 2001-2004
	Bachelor of Science (Biochemistry)
	(First Class Honors)
	Mahidol University, 2008-2014
	Doctor of Philosophy (Molecular
	Genetics and Genetic Engineering)
RESEARCH GRANTS	Royal Golden Jubilee Scholarship
	(PHD/0268/2549)
HOME ADDRESS	316/25 Wachiraprakarn Rd., Bangplasoi,
	Muang, Chonburi, Thailand 20000
	Tel. +66(38)271758, +66(7)1001686
	E-mail: chitwadeeae@gmail.com
PUBLICATION / PRESENTATION	Phithakrotchanakoon C, Champreda V,
	Aiba S, Pootanakit K, Tanapongpipat S.
	(2013) Engineered Escherichia coli for
	Short-Chain-Length Medium-Chain-Length
	Polyhydroxyalkanoate copolymer
	biosynthesis from glycerol and
	dodecanoate. Biosci Biotechnol Biochem.
	77(6): 1262-68.
	Phithakrotchanakoon C, Champreda V,
	Aiba S, Pootanakit K, Tanapongpipat S.
	(2014) Production of
	polyhydroxyalkanoates from crude glycerol

using recombinant Escherichia coli. J Polym Environ (in press). Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2013, 5-7 April) Engineered Escherichia *coli* for biosynthesis of polyhydroxyalkanoates bio-plastic from glycerol. Paper presented at The RGJ-Ph.D. Congress XIV, Pattaya, Thailand (outstanding poster presentation award) Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2012, 25-29 Nov) Bioproduction of polyhydroxyalkanoate bio-plastic from byproduct of biodiesel by engineered Escherichia coli. Paper presented at 13th FAOBMB congress, Bangkok, Thailand Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2011, 20-21 Dec) Bioproduction of Short-Chain-Length-co-Medium-Chain-Length-Polyhydroxyalkanoates bioplastic from crude glycerol by engineered Escherichia coli. Paper presented at The third Thai-Japan bioplastics and biobased materials symposium, Bangkok, Thailand Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2010, 20-22 Oct) Polyhydroxyalkanoate bioplastics production from crude glycerol by recombinant Escherichia coli. Paper

presented at The Thai Society of Biotechnology 2010, Trang, Thailand Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2009, 14 Dec) Polyhydroxyalkanoate bioplastic production from crude glycerol by recombinant Escherichia coli using metabolic engineering approach. Paper presented at The young scientist seminar bioresources: their potentials and applications, Bangkok, Thailand Phithakrotchanakoon C, Champreda V, Aiba S, Pootanakit K, Tanapongpipat S. (2009, 2 Sep) Polyhydroxyalkanoate bioplastics production from crude glycerol by recombinant Escherichia coli using metabolic engineering approach. Paper presented at The First Thai-Japan bioplastic and biobased material symposium, Bangkok, Thailand