PROCESS DEVELOPMENT FOR THE PRODUCTION AND SEPARATION OF MEDIUM-CHAIN-LENGTH POLY(3-HYDROXYALKANOATES) BY *PSEUDOMONAS PUTIDA* KT2440

by

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Abstract

A series of medium-chain-length poly(3-hydroxyalkanoates) (MCL-PHAs) with enriched 3-hydroxynonanoate (HN) content (up to 95.8 mol% compared to 68.6 mol% without acrylic acid) or 3-hydroxyoctanoate (HO) content (up to 97.5 mol% compared to 88.0 mol% without acrylic acid) was produced in continuous culture by co-feeding fatty acid and glucose plus inhibiting fatty acid β-oxidation using acrylic acid. Using a similar feeding strategy in fed-batch fermentation, similar monomeric compositions but a higher biomass concentration and PHA content could be obtained. However, at a lower growth rate (0.15 h^{-1} vs. 0.25 h^{-1}), the biomass concentration and PHA content could be greatly enhanced from 17.1 to 71.4 g L^{-1} and from 64.4 to 75.5%, respectively, while the HN content decreased slightly from 92.2 to 88.9 mol%. PHAs produced under acrylic acid inhibition possessed improved physical properties including a higher melting point, faster crystallization rate, and greater tensile strength at break and Young's modulus.

Two recovery methods were developed for the recovery of MCL-PHA from *Pseudomonas putida* KT2440. One applied acetone extraction which was capable of recovering all the PHA from the cells with a purity of 91.6% and no detectable polymer molecular weight loss using Soxhlet extraction. Further purification was achieved by redissolving in acetone and reprecipitating in cold methanol. The other method used sodium hydroxide to solubilize the non-PHA cellular material. PHA purity of about 85% was obtained from a biomass containing 65.6% PHA after treatment with 0.2 N NaOH at 22 $\pm 1^{o}$C for 2 h or with 0.1 N at 80 $\pm 1^{o}$C for 15 min. However, a treatment at 22 $\pm 1^{o}$C followed by a second NaOH treatment at 80 $\pm 1^{o}$C resulted in higher PHA purity (94.7%) with a recovery efficiency of 88%. Under these conditions, NaOH digestion had a negligible effect on PHA molecular weight.

Co-Authorship

The author was supervised by Dr. Bruce A Ramsay and Dr. Juliana A Ramsay, who contributed to the preparation of all manuscripts presented in this thesis. Acknowledgement also goes to Dr. Zhiyong Sun who was involved in discussions for developing the fermentation process for PHA production and revisions to Chapters 3 and 4.

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List of Abbreviations and Symbols

AA	Acrylic acid
AcCoA	Acetyl co-enzyme A
ADP	Adenosine 5’ diphosphate
ATP	Adenosine 5’ triphosphate
CoA	Co-enzyme A
CO_2	Carbon dioxide
CPR	CO_2 production rate
D	Dilution rate (h^{-1}, D equals to μ at steady state in chemostat fermentation)
DCW	Dry cell weight
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
EDTA	Ethylene diamine tetraacetic acid
E4P	Erythrose-4-phosphate
FA	Fatty acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide, reduced
F6P	Fructose-6-phosphate
f_{NA}	Mass fraction of nonanoic acid in the total carbon sources
f_G	Mass fraction of glucose in the total carbon source
G	Glucose
GC	Gas chromatography
GOX	Glyoxylate
GPC	Gel permeation chromatography
G3P	Glyceraldehyde 3-phosphate
G6P	Glucose-6-phosphate
HB	3-hydroxybutyrate
HD	3-hydroxydecanoate
HDD	3-hydroxydodecanoate
HHp	3-hydroxyhepanoate
HHx	3-hydroxyhexanoate
HN	3-hydroxynonanoate
HN (=)	3-hydroxynonenoate

HO	3-hydroxyoctanoate
HUD (=)	3-hydroxyundecenoate
HV	3-hydroxyvalerate
IsoCiT	Isocitrate
KG	Ketoglutarate
MAL	Malate
MCL	Medium-chain-length
Mw	Molecular weight
NA	Nonanoic acid
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NADH	Nicotinamide adenine dinucleotide (phosphate), reduced
OA	Octanoic acid
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
PHA	Poly(3-hydroxyalkanoate)
PHA-n	PHA with n carbons, where n is the number of carbons in the fatty acid substrate
PHA-n-2	PHA with n-2 carbons
PHA-n-4	PHA with n-4 carbons
PHB	Poly(3-hydroxybutyric acid)
P(HB-HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(HB-HHx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(HN-HHp)	Poly(3-hydroxynonanoate-co-3-hydroxyheptanoate), also known as PHN
P(HO-HHx)	Poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate), also known as PHO
PYR	Pyruvate
r_{bio}	Residual biomass production rate (per gram of residual biomass, h^{-1})
r_{FA}	Fatty acid consumption rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
r_G	Glucose consumption rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
r_{PHA}	PHA synthetic rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
Ru5P	Ribulose-5-phosphate
R5P	Ribose-5-phosphate
rpm	Revolutions per minute
SCL	Short-chain-length
SDS	Sodium dodecyl sulfate
S_t	Cumulative mass of carbon substrates (g) to be fed at time t (h)

SUC	Succinate
SucCoA	Succinate co-enzyme A
S7P	Sedoheptulose-7-phosphate
THF	Tetrahydrofuran
T_g	Glass transition temperature
TGA	Thermogravimetric analysis
TKN	Total Kjeldahl Nitrogen analysis
T_m	Melting point
UV	Ultraviolet
X	Biomass (g)
X_r	Residual biomass (g)
X_t	Biomass (g) at time t (h)
X_0	Estimated biomass (g) at the beginning of the feeding
X5P	Xylulose-5-phosphate
$Y_{X/C}$	Yield of biomass from total carbon substrate
$Y_{PHA/NA}$	Yield of PHA from nonanoic acid
$Y_{PHA/OA}$	Yield of PHA from octanoic acid
μ	Specific growth rate (h^{-1})
σ_b	Tensile stress at break (MPa)
E	Young's modulus (MPa)
ϵ_b	Elongation at break (%)
3PG	3-phosphoglycerate
6PG	6-P-gluconate
7POHH	7-phenoxy-hydroxyheptanoic acid
9POHN	9-phenoxy-hydroxynonanoic acid
11-POU	11-phenoxyundecanoic acid
vvm	The volumetric air flow rate (ml min^{-1}) divided by the volume of the fermentor

Chapter 1

Introduction

1.1 Background

The 20th century has witnessed the growth of petrochemical-based plastics. Their global annual production increased from 2 million tons in the 1950s to 120 million tons in 2000 (Meyer and Keurentjes, 2005). They have a wide range of applications from food wrapping to house building and they have become an indispensable part of daily life. However, starting in the 1970s, concern about the future availability of petroleum has expanded the development of materials derived from renewable resources. Furthermore, the disposal of large quantities of environmentally persistent petro-based plastics has become a problem of modern life. None of the current methods such as land filling, recycling and incineration have proved satisfactory. Alternatives that are both produced from renewable resources and are compostable are required for sustainable development. One of the most studied compostable polyesters is poly(3-hydroxybutyrate) (PHB), a member of the poly(3-hydroxyalkanoate) (PHA) family. These materials are accumulated by a wide variety of bacteria from a wide range of renewable resources such as carbohydrates and vegetable oils. After decades of research, they show tremendous potential.

The study of PHA began in 1926 when Lemoigne discovered PHB in *Bacillus megaterium* (Lemoigne, 1926). Researchers began to realize that PHAs are a series of homopolymers and copolymers after more hydroxyalkanoate constituents were discovered (Findlay and White, 1983). Ninety-one PHA constituents had been reported

by 1995 (Steinbuchel and Valentin, 1995) and about 150 by 2003 (Steinbuchel and Lutke-Eversloh, 2003). Most variants have the same backbone and differ only in the monomer side chain, R (Figure 1-1). The side chain may contain only one or up to fifteen carbons and may also contain functional groups. Its length and structure play a dominant role in determining polymer properties.

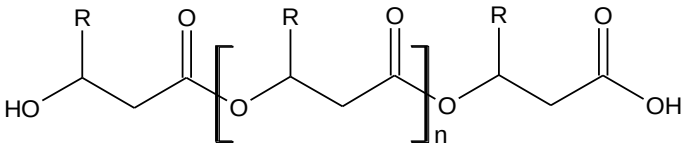

Figure 1-1 Chemical structure of polyhydroxyalkanoate (Adapted from Steinbuchel and Valentin, 1995).

PHAs with monomers containing up to five carbons are called short-chain-length (SCL) PHAs. They were discovered before the other types because their accumulation is common to many bacteria. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(HB-HV), is the second most studied PHA after PHB. It was first commercialized by Imperial Chemical Industries Inc. in the UK under the trade name Biopol® in the 1980s. These polymers are typically highly crystalline, possess comparatively high melting points (e.g. 180^{o}C for PHB, and 140^{o}C for P(HB-HV) containing 25% of HV) and in many ways resemble conventional thermoplastics such as polypropylene (Holmes, 1985). The second class of PHA is medium-chain-length (MCL) PHA, containing six to fourteen carbons in their repeating units. MCL-PHA has never been commercially available as there are fewer bacteria which can produce the polymer and the productivity was comparatively low (Sun et al., 2007). Unlike the SCL-PHA, MCL-PHAs are thermoelastic and much less crystalline (Marchessault et al., 2001). Their side chains offer an opportunity to graft functional groups or to perform other chemical modifications. They can be used in

applications such as coatings (Van der Walle et al., 1999), controlled release carriers (Kurth et al., 2002), and in tissue engineering (Sodian et al., 2000). The third class of PHA, SCL-MCL-PHA, is a family of hybrid copolymers of HB and the MCL-PHA monomers. Procter and Gamble developed these copolymers under the trademark of NodaX. By varying the monomeric composition, the polymer can have a melting point between 80 to 164^{o}C (Green, 2001).

Although both SCL-PHA and SCL-MCL-PHA have been commercially developed, MCL-PHA still remains at the research and development stage. One of the major obstacles lies in its processability. The melting point of MCL-PHAs are comparatively low, mostly ranging from 40 to 60^{o}C (Kim and Lenz, 2001). The polymer becomes soft and sticky at temperatures close to its melting point, which could cause problems during its storage and transportation, and hence might limit its applications. Studies have shown that PHA properties, e.g. melting point, are closely related to its monomeric composition (Gross et al., 1989). MCL-PHA also has a slow crystallization rate and a low degree of crystallinity. Such characteristics, which are largely due to monomers with different length side chains, make MCL-PHA unsuitable for conventional thermoplastic processing. MCL-PHAs with unusual properties have been obtained by genetically modifying strains (Liu and Chen, 2007; Ouyang et al., 2007). Fermentation strategies can also affect the PHA biosynthetic pathway and so modify the monomeric composition (Du and Yu, 2002; Aldor and Keasling, 2003). However, little has been achieved in the control of PHA monomeric composition during production of MCL-PHA.

MCL-PHA production also faces challenges in the development of an automated process because of poor substrate solubility and toxicity at low concentrations (Sun et al.,

2007). High substrate cost, low product yield and the difficulty in recovery also increase production cost. The recovery of MCL-PHA has been much less studied than that of SCL-PHA. Although SCL-PHA separation processes have been well developed, they cannot be directly applied to MCL-PHA because of the differences in the polymer properties and the producing strains.

1.2 Chapters and objectives

This thesis focused on developing fermentation strategies for the production of MCL-PHAs with higher melting points and developing methods for the recovery of the MCL-PHA polymers from the bacterial cells.

The thesis consists of seven chapters. Chapters one and two are the introduction and literature review, respectively. Chapters three to six correspond to four specific objectives. The objective of Chapter three was to produce MCL-PHAs with enriched dominant monomer content in chemostat culture and to study its effect on melting point and mechanical properties.

Continuous cultivation is effective in the investigation of microbial physiology and growth kinetics, but it is less practical at industrial scale production due to its high potential for microbial contamination. The objective of Chapter four was to develop fed-batch fermentation processes for the production of MCL-PHA with elevated dominant monomer content.

Chapter five aimed at establishing a lab-scale MCL-PHA recovery process using common organic solvents. Acetone was chosen as MCL-PHA extraction solvent while methanol was used for biomass pretreatment and PHA precipitation.

The objective of Chapter six was to develop a recovery method suitable for commercial-scale production mainly based on the use of inorganic reagents. MCL-PHA separation by sodium hydroxide digestion of non-PHA cellular materials was therefore studied and described.

Finally, Chapter seven gives conclusions and recommends future work.

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Chapter 2
Literature Review

2.1 Biosynthesis of MCL-PHA

2.1.1 MCL-PHA-producing microorganisms

Many bacteria accumulate SCL-PHA, possibly as a carbon and energy reserve (Byrom, 1987; Anderson et al., 1990). In contrast, MCL-PHA synthesis is restricted to *Pseudomonas* rRNA homology group I, for example *P. aeruginosa*, *P. chlororaphis*, *P. putida*, *P. syringae* and some *P. fluorescens* (Diard et al., 2002). It is interesting that the *P. oleovorans* group accumulates SCL-PHA on octanoate. A well known strain, formerly *P. oleovorans* GPo1, which is capable of MCL-PHA accumulation, has been reclassified in the *P. putida* complex and is now known as *P. putida* GPo1 (Van Beilen et al., 2001). *P. putida* strains are the most frequently studied bacteria in MCL-PHA production.

2.1.2 Dependence of MCL-PHA synthesis on carbon sources

MCL-PHA-producing bacteria can accumulate the polyester from both structurally related substrates, such as fatty acids or their salts (Durner et al., 2001; Sun et al., 2007b), aliphatic alkanes (De Smet et al., 1983; Hazenberg and Witholt, 1997), and alkenes (Kim et al., 1995), and non-structurally related carbon compounds, such as glucose and sucrose (Sanchez et al., 2003; Timm and Steinbuchel, 1990). MCL-PHA synthases typically have relatively broad substrate specificity, though all the precursors must be in the R-configuration (Steinbuchel and Valentin, 1995). Precursors containing six to fourteen carbons can be metabolized with a preference for eight or nine carbon substrates (Lageveen et al., 1988; Gross et al., 1989; Kang et al., 2001). Therefore, MCL-PHAs are almost always copolymers. Their monomeric composition depends greatly on the strain

and the carbon substrate (Table 2-1) but for a given microorganism and substrate, the polymer composition usually varies little.

Table 2-1 MCL-PHAs produced by *Pseudomonas* from different substrates

Bacteria	Carbon source	Major HA monomers	Reference
P. putida KT2442	Glucose	HD, HO	Huijberts et al., 1992
P. putida IPT046	Glucose + Fructose	HD, HO	Sanchez et al., 2003
P. putida KT2442	Oleic acid	HD, HO, HHx	Lee et al., 2000
P. putida GPo1	10-undecenoic acid	HUD (=), HN (=)	Kim et al., 1995
P. putida KT2440	Nonanoic acid	HN, HHp	Sun et al., 2007b

HD, 3-hydroxydecanoate; HO, 3-hydroxyoctanoate; HHp, 3-hydroxyhepanoate; HHx, 3-hydroxyhexanoate; HUD (=), 3-hydroxyundecenoate; HN (=), 3-hydroxynonenoate

Monomer chain length is closely related to the chain length of the carbon substrate. For example, *Pseudomonas putida* GPo1 grown on octanoate, produces poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate-co-3-hydroxydecanoate) with HO as the dominant monomer (Witholt et al., 1994). When long-chain-length fatty acid is fed as the carbon source, the monomeric composition becomes more complex. For instance, oleic acid, coconut oil, or tallow produced polymers containing five or more different monomers (Lee et al., 2000; Thakor et al., 2005; Cromwick et al., 1996). The same effect can occur if a mixture of substrates is fed. Structurally unrelated substrates such as carbohydrates can be used by some pseudomonas strains such as *Pseudomonas putida* KT2442 to synthesize MCL-PHA composed mainly of 3-hydroxydecanoate (HD) and HO (Huijberts et al., 1994). This is attributed to 3-hydroxyacyl-CoA-ACP transferase (PhaG) which diverts the intermediates of *de novo* fatty acid synthesis to PHA formation (Rehm et al., 1998).

Structurally related substrates which usually result in higher PHA yields (gram PHA synthesized per gram substrate consumed) than structurally unrelated substrates are normally more expensive. To reduce production costs, the two types of substrates can be used together, allowing the more structurally related substrate to be primarily diverted to PHA accumulation. When *P. putida* KT2440 was grown on a mixture of nonanoic acid and glucose, the yield of PHA from nonanoic acid almost doubled from 0.254 mol mol^{-1} to 0.450 mol mol^{-1} while the yield of PHA from glucose decreased (Sun et al., 2007a).

The solubility and toxicity of the carbon sources are important in process development. Hydrocarbons such as octane, existing as a separate organic phase, exhibit little toxicity but may impose mass transfer limitation (Hazenberg and Witholt, 1997). Alkanoic acids are more miscible while alkanoate salts are highly water soluble, but most are toxic above a certain concentration. Nonanoic acid was found to inhibit the growth of *P. putida* KT2440 at 3~4 g L^{-1} (Sun et al., 2006) while octanoate inhibited *P. putida* GPo1 at 4.65 g L^{-1} (Ramsay et al., 1991). Carbohydrates, such as glucose, did not affect bacterial growth at concentrations as high as 40 g L^{-1} (Kim et al., 1996).

2.1.3 Metabolic routes for MCL-PHA synthesis

MCL-PHA synthase requires R-3-hydroxyacyl coenzyme A thioester (R-3-hydroxyacyl-CoA) for PHA polymerization (Nomura and Taguchi, 2007). ^{1}H and ^{13}C NMR studies have shown that fatty acid β-oxidation and fatty acid *de novo* synthesis are the two major metabolic pathways which provide intermediates for MCL-PHA precursors (Eggink et al., 1992). Fatty acid β-oxidation is the main route for the conversion of fatty acids, alkanes, and alkenes to MCL-PHA precursors, while the fatty acid *de novo*

synthesis pathway is mainly responsible for MCL-PHA accumulation from structurally unrelated carbon compounds.

Alkanes and alkenes must be first converted to fatty acids by alkane oxidation before they can undergo β-oxidation. In β-oxidation, fatty acids are oxidized and acetyl-CoA units are released for energy generation or lipid synthesis (Figure 2-1). The remaining acyl-CoA shortened by two carbons can enter the β-oxidation cycle again. Therefore, intermediates differing by two methyl groups in each cycle may be used for PHA synthesis resulting in the copolymeric character of MCL-PHA (Lageveen et al., 1988).

There are three possible intermediates that may be used to synthesize R-3-hydroxyacyl-CoA (Vanderleij and Witholt, 1995). One is trans-2-enoyl-CoA catalyzed by enoyl-CoA hydratase (Figure 2-1, reaction 9). The gene encoding this enzyme has been found in *Aeromonas caviae* (Fukui and Doi, 1997) and *P. aeruginosa* (Tsuge et al., 2000). A second possible route, though there is little evidence, is from (S)- hydroxyacyl-CoA to its (R)-isomer catalyzed by 3-hydroxyacyl-CoA epimerase (reaction 10). Another route is from 3-ketoacyl-CoA by reductase (reaction 11, Taguchi et al., 1999). At least one of these pathways likely exists in a recombinant *E. coli* harboring *phaC1* gene from *P. aeruginosa* since enhanced MCL-PHA accumulation was attained when the key enzyme (3-ketoacyl-CoA thiolase in reaction 8) was inhibited and the final step of β-oxidation was impaired (Qi et al., 1998).

Fatty acid de novo synthesis is less efficient than fatty acid β-oxidation in PHA synthesis but it does provide PHA precursors when *P. putida* is grown on glucose (Huijberts et al., 1992; Huijberts et al., 1994). The gene *phaG* encodes acyl transferases which converts (R)-3-hydroxyacyl-ACP to (R)-3-hydroxyacyl-CoA (reaction 13) and

connects fatty acid synthesis to PHA formation (Rehm et al., 1998; Hoffmann et al., 2000a; Hoffmann et al., 2000b; Matsumoto et al., 2001). Unlike β-oxidation, 3-ketoacyl synthase in fatty acid synthesis increases the precursor chain length by incorporating acetyl-ACP, a two-carbon unit allowing the formation of longer monomer components from short-chain-length substrates.

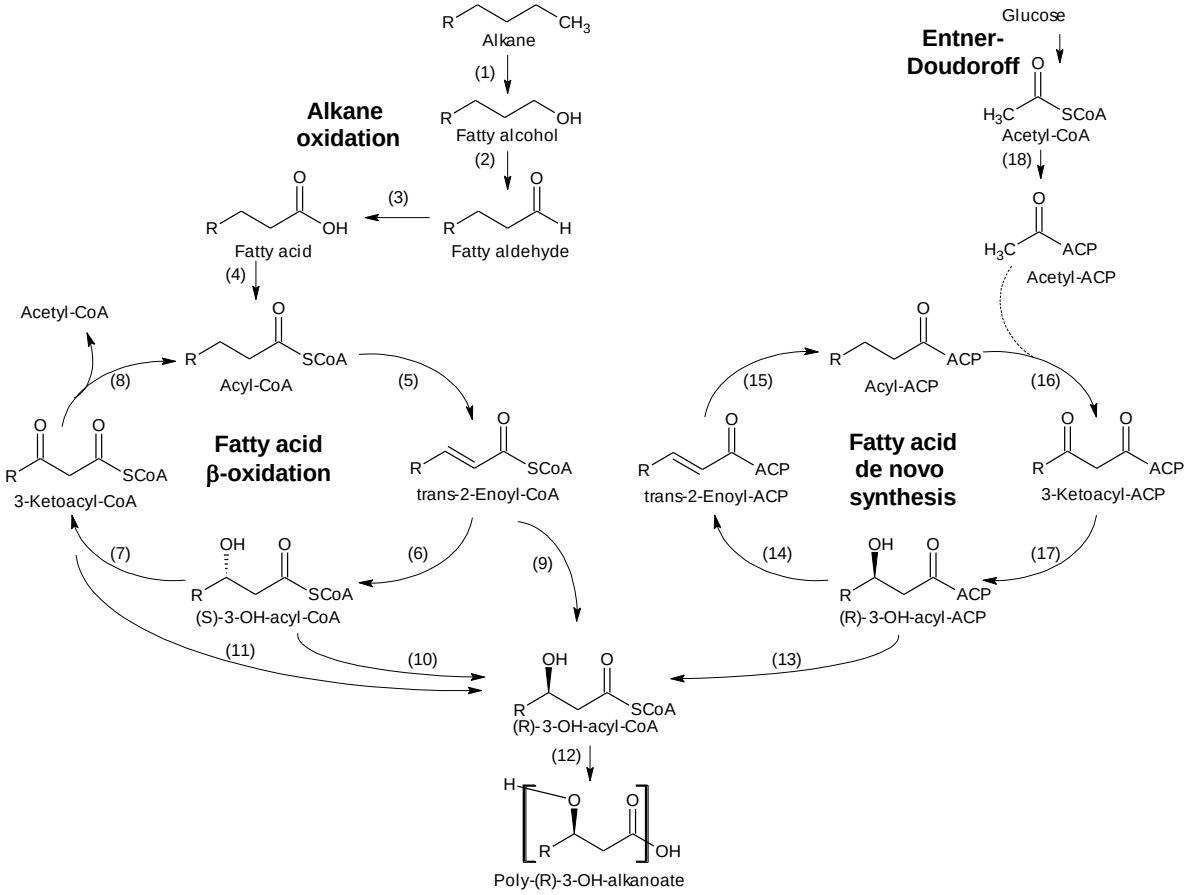

Figure 2-1 Major pathways involved in MCL-PHA synthesis in pseudomonads. The confirmed or postulated enzymes of each step are numbered as following: (1) alkane hydroxylase (2) alcohol dehydrogenase (3) aldehyde dehydrogenase (4) thiokinase (5) acyl-CoA dehydrogenase (6) enoyl-CoA hydratase or crotonase (7) (S)-3-hydroxyacyl-CoA dehydrogenase (8) 3-ketoacyl thiolase (9) enoyl-CoA hydratase (10) 3-hydroxyacyl-CoA epimerase (11) ketoacyl-CoA reductase (12) PHA polymerase (13) (R)-3-hydroxyacyl (ACP-CoA) transferase (14) 3-hydroxyacyl-ACP dehydratase (15) enoyl-ACP reductase (16) 3-ketoacyl synthase (17) ketoacyl-ACP reductase (18) acetyl-CoA:ACP transacylase. (Adapted from Vanderleij and Witholt, 1995).

2.1.4 Control of monomeric composition

Due to the complexity of the metabolic pathways and the broad specificity of PHA synthases, naturally occurring MCL-PHAs are almost all copolymers except for six-carbon and seven-carbon MCL-PHA homopolymers which are accumulated in very low content (Table 2-2). The minor monomer content increases if longer chain length substrates are fed. This is because substrates with long backbones are continuously cleaved by β-oxidation and most of the intermediates can be utilized for PHA formation.

Gene knockout or amplification has been used to manipulate PHA composition. Knockout of the *fadA* and *fadB* genes encoding for 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase (responsible for reaction 8 and 7, respectively, in Figure 2-1) was found to be effective in increasing the metabolic flux from β-oxidation intermediates to PHA synthesis in *P. putida* KTOY06 (Ouyang et al., 2007; Liu and Chen, 2007) and *P. putida* KCTC1639 (Vo et al., 2007), with a significant increase in the fraction of the longer chain length monomers. The β-oxidation impaired *P. putida* KTOY06 (both *fadA* and *fadB* genes were knocked out) produced a PHA containing 41 mol% 3-hydroxydodecanoate while the wild type strain only produced 7.5 mol% when dodecanoate was the sole carbon source in shake flask culture (Ouyang et al., 2007). When the *fadA* and *fadB* genes were knocked out and the PHA synthase gene amplified in a recombinant *Escherichia coli* harboring the *Pseudomonas sp*. 61-3 PHA synthase gene ($phaC2_{Ps}$), the recombinant strain produced PHA containing up to 80 mol% 3-hydroxydecanoate and 48 mol% 3-hydroxydodecanoate from sodium decanoate and sodium dodecanoate, respectively (Park et al., 2003).

Table 2-2 Monomeric composition of MCL-PHAs produced from alkanes or alkanoic acids

Bacteria	Culture scale	Carbon source	% of PHA (g/100g DCW*)	Monomeric composition of PHA**					Reference
				HHx	HHp	HO	HN	HD	
P. putida Gpo1	0.5-1 L	Hexane	2.0	100					Lageveen et al., 1988
		Heptane	11.4		100				
		Octane	25.3	11		89			
		Nonane	24.3		37		63		
P. putida Gpo1	50 ml	Hexanoate	3.3	95		5			Huisman et al., 1989
		Heptanoate	2.3		100				
		Octanoate	8.7	8		91		1	
		Nonanoate	9.1		35		65		

* DCW stands for dry cell weight;
** HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyhepanoate; HO, 3-hydroxyoctanoate; HN, 3-hydroxynonanoate HD, 3-hydroxydecanoate

However, the use of genetic modification has limited flexibility since the monomeric composition varies little when the strain and the substrate are fixed.

Enzyme inhibitors have also been used to affect PHA synthesis. As early as 1964, acrylic acid was reported to impair β-oxidation by inhibiting ketothiolase (Thijsse, 1964), the key enzyme in the last step of the oxidation cycle, and led to the accumulation of free fatty acids in *Pseudomonas* (Strain 473) from substrates such as hexane. Acrylic acid or acrylic acid was found to enhance SCL-MCL-PHA accumulation in *Ralstonia eutropha* (Green et al., 2002), a genetically engineered *Aeromonas hydrophila* 4AK4 (Han et al., 2004) and a recombinant *Escherichia coli* (Lu et al., 2003). Up to a 30-fold increase in MCL-PHA content was achieved through use of acrylic acid on recombinant *E. coli* strains harboring MCL-PHA synthase genes from *P. aeruginosa*, but there was no mention of any change in PHA monomeric composition (Qi et al., 1998).

While acrylic acid efficiently channeled intermediates towards PHA synthesis, the impairment of β-oxidation decreased energy production. Severe inhibition normally resulted in poor growth and no PHA synthesis if a fatty acid was the sole carbon source (Huijberts et al., 1994).Therefore, an additional substrate should be provided as an energy source. It must not require β-oxidation nor lead to the accumulation of undesired PHA monomers.

Recently, Choi et al. (2009) reported a higher accumulation of long-side-chain aromatic monomers in *Pseudomonas fluorescens* BM07 when co-feeding with 50 mM fructose and 5 mM 11-phenoxyundecanoic acid (11-POU) in the presence of 1.5 mM salicylic acid as an inhibitor. The PHA (30% of 1.5 g L^{-1} biomass produced) had seven to eight monomer constituents derived from both substrates but contained a total of 61mol%

of 7-phenoxy-hydroxyheptanoic acid (7POHH) and 9-phenoxy-hydroxy-nonanoic acid (9POHN), compared to a total of 29 mol% 7POHH and 9POHN in the control without salicylic acid. It was suggested that acrylic acid and salicylic acid had a similar function inhibiting β-oxidation pathway.

2.2 Properties of MCL-PHA

2.2.1 General properties

MCL-PHAs are biodegradable, have low gas permeability and good water resistance. Their weight average molecular weight is generally within 60 ~ 400 kDa (Witholt and Kessler, 1999) with a density close to 1 g cm^{-3} (Marchessault et al., 1990). For the sake of convenience, they are often named based on the dominant monomer constituent in the polymer. For example, PHA reported in the literature as poly(3-hydroxyoctanoate) is actually a copolymer containing at least a minor amount of 3-hydroxyhexanoate and often other monomers as well.

Crystallinity affects both thermal and mechanical properties of a thermoplastic. The crystallization rate of MCL-PHA is generally very slow. It was reported that seven weeks were required to obtain maximum crystallinity of poly(3-hydroxyoctanoate) at 20^{o}C while sixteen weeks were required at 5^{o}C (Gagnon et al., 1992). It took several hours for poly(3-hydroxynonanoate) to achieve a maximum crystallinity of about 25% (Marchessault et al., 1990). X-ray diffraction patterns suggested that the polymer crystallized as a 2_1 helix in an orthorhombic lattice with two molecules per unit cell. The long side chains showed little tendency to crystallize, however both the backbone and the side chains are involved in a layered packing order (Figure 2-2) (Gross et al., 1989; Marchessault et al., 1990; Dufresne et al., 2001).

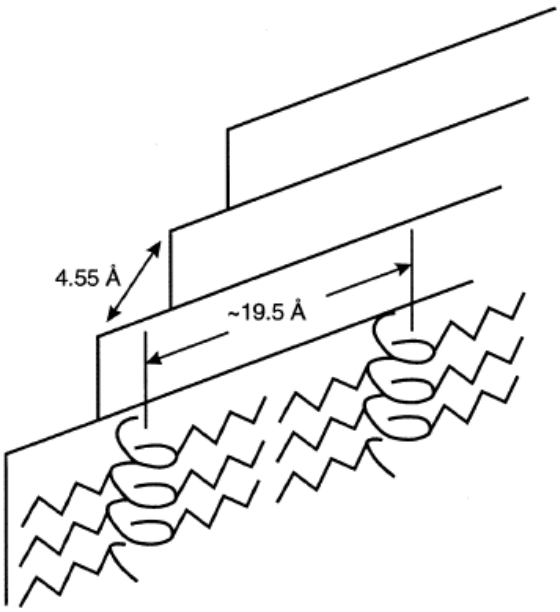

Figure 2-2 The sheet-like molecular arrangement with "all trans" side chains. The interplanar distance between sheets is 4.55 Å and the intraplanar distance from helix to helix is 19.5 Å for poly(3-hydroxyoctanoate) (Dufresne et al., 2001).

MCL-PHAs are thermoplastic elastomers. Table 2-3 shows the mechanical and thermal properties of several MCL-PHAs. Their melting temperatures range approximately from 40 to 60^{o}C. At temperatures close to or above the melting point, the polymer becomes amorphous and sticky. The low melting point MCL-PHAs soften at ambient temperature. This may make processing difficult and severely limit potential applications. Due to the lack of material availability, MCL-PHA property data are limited and may vary depending on the method of treatment and analysis by different researchers.

2.2.2 Dependence of melting temperature and crystallinity on side-chain length

In contrast to MCL-PHA, the SCL-PHA homopolymer, PHB, has a high degree of crystallinity of at least 70% and a melting temperature around 180^{o}C (Holmes, 1984; Holmes, 1988). Increasing 3-hydroxyvalerate content from 0 to 43% decreases the

melting point from 180 to 79^{o}C. This improves its processability greatly since PHAs degrade at higher temperatures (Doi et al., 1986). When only 25 mol% 3-hydroxyhexanoate was introduced into PHB as a second monomer constituent, the crystallinity and melting point decreased to 18% and 52^{o}C, respectively (Doi et al., 1995; Green, 2001). Ouyang et al. (2007) reported that the T_m increased from 53 to 65^{o}C and ΔH_m increased from 18 to 28 J g^{-1} when the 3-hydroxydodecanoate fraction of an MCL-PHA was increased by feeding a genetically modified *Pseudomonas putida* strain on dodecanoate. The melting point of MCL-PHA seems to increase as the length of the side chains increase (Table 2-3).

2.3 Recovery of MCL-PHA from microorganisms

2.3.1 PHA-producing bacteria: cell wall structure and components

PHA is accumulated intracellularly and can be observed as refractive granules under a phase contrast microscope and as electron-transparent regions in electron microscopy (Figure 2-3). The granules are generally spherical with a surface mainly consisting of phospholipids and proteins related to PHA synthesis and degradation.

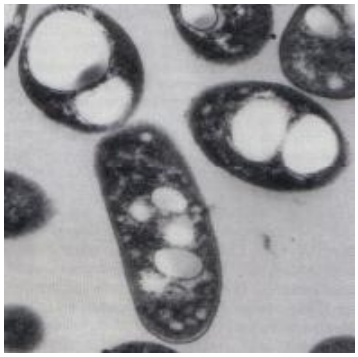

Figure 2-3 Thin-section electron microscopy of *P. oleovorans* (reclassified as *P. putida*) containing PHA granules (Lageveen et al., 1988).

Table 2-3 Important thermal and mechanical properties of typical MCL-PHAs

Organism	Substrate	Monomeric composition	Mw (KDa)	Dispersity (Mw/Mn)	Tg (^{o}C)	Tm (^{o}C)	Young's modulus (MPa)	Tensile strength (MPa)	Elongation to break (%)	Reference
P. oleovorans	n-hexane	HHx (83%), HO	330	1.8	-25.8					Preusting et al., 1990
P. oleovorans	heptanoate	HHp (86%), HN, HV	360	3.0	-33	45				Gross et al., 1989
P. oleovorans	octanoate	HO (75%), HHx	160	3.0	-36	61				Gross et al., 1989
P. oleovorans	octanoate	HO (86%), HHx			-35	61	3-15	6-10	300-450	Gagnon et al., 1992
P. putida KTOY06	dodecanoate	HDD (39%), HD, HO, HHx	157	1.45	-43	65	11.5	16.3	125	Ouyang et al., 2007
P. putida KTOY06	tetradecanoic acid	HTD (49%), HDD, HD, HO, HHx	95	1.43	-40	66.8	34	3.15	107.7	Liu and Chen, 2007

HV, 3-hydroxyvalerate; HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyheptanoate; HO, 3-hydroxyoctanoate; HN, 3-hydroxynonanoate; HD, 3-hydroxydecanoate; HDD, 3-hydroxydodecanoate

There are many other cellular components which should be eliminated from the final PHA product (Table 2-4). The bacterial cell wall is the first and greatest barrier to PHA separation. The *Pseudomonas* (Gram-negative) cell wall consists of three layers from the outside to inside: the outer membrane, peptidoglycan, and the inner membrane (Figure 2-4). Both outer and inner membranes are lipid bilayers with various proteins embedded in them. Lipopolysaccharides (LPS) found in the outer membrane are endotoxins that may cause a fever when introduced into humans or animals and is a concern in medical applications. Peptidoglycan is a skeleton structure accounting for about 10% of the cell wall in Gram-negative bacteria. It has a glycan backbone (a repeat unit of N-acetyl-glucosamine linked to N-acetylmuramic acid) and is strengthened by crosslinking of the amino acid side chain with its tetrapeptide neighbours. Since it is an essential part of the cell wall structure and difficult to degrade, analysis of residual peptidoglycan by determining the N-acetylmuramic acid content could be useful in confirming PHA purity.

Table 2-4 Chemical composition of a typical Gram-negative prokaryotic cell (Madigan et al., 2000)

Cell component	% dry weight
Macromolecules	96
Proteins	55
Polysaccharide	5
Lipids	9.1
Lipopolysaccharide	3.4
Nucleic acids	23.6
Monomers	3.0
Inorganic ions	1
Total	100

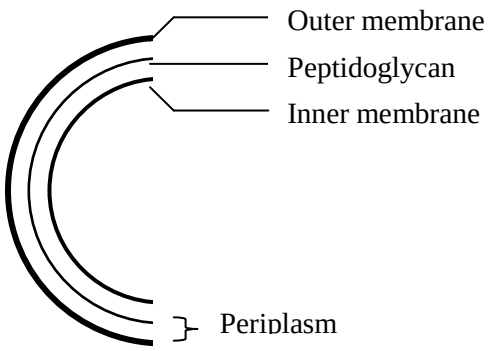

Figure 2-4 Schematic diagram of Gram-negative cell wall.

2.3.2 Recovery process overview

A PHA recovery process (Figure 2-5) typically begins with separation of the cells from the fermentation broth by centrifugation or filtration. Sometimes a biomass pretreatment step is necessary to facilitate subsequent procedures to save chemicals or eliminate cellular components. For example, aqueous washing of the biomass can be used to remove salts, or solvents may be applied to remove lipids. PHA recovery can be achieved by solvent extraction of the polymer, chemical digestion of the non-PHA biomass or mechanical disruption of the cell wall and inner membrane. Further purification may be required to obtain a desired PHA purity. Apart from recovery conditions, practicality, cost and the effect on polymer properties must be considered when developing a separation process.

2.3.3 Recovery process based on solvent extraction

Solvents in PHA extraction must penetrate the cell envelope, and dissolve the polymer leaving the cell debris in suspension. It is the most commonly used method for small-scale PHA recovery. Most PHA scientific and patent literature concerning PHA extraction processes deals with SCL-PHA such as PHB. Because some cell components can dissolve in the extraction solvents and are recovered with PHB, pre-treatment with a

poor PHB solvent (e.g. acetone) has been used to remove water, lipids and other cell components (Baptist, 1962). Poor PHB solvents also include hexane, octane, methanol, ethanol and ether (Coty, 1966; Kurdikar et al, 2000). Chlorinated solvents, especially chloroform and methylene chloride, are the most commonly used extracting solvents (Table 2-5). After the extraction, PHA may be recovered by either solvent evaporation or precipitation in a poor PHA solvent.

Soxhlet extraction uses less solvent and enhances the recovery yield since the solvent is refluxed allowing the biomass to be repeatedly extracted with fresh solvent. For example, 50% PHB was obtained from lyophilized *R. eutrophus* biomass using chloroform by Soxhlet extraction compared to only 27% by direct solubilization under ambient conditions (Ramsay et al., 1994). Generally, the efficiency of solvent extraction under ambient conditions depends on the PHA content in biomass. A large volume of solvent is required during extraction because the solution becomes viscous as PHA concentration increases.

Loss of polymer molecular weight is a concern with some solvents (e.g. chloroform) during prolonged extraction at high temperature (Traussnig et al, 1990). The degradation may be attributed to several reasons. One is the stability of PHA in different solvents. It has been reported that the intrinsic viscosity of PHB decreased by 37% in a chloroform-methanol mixture over 20 days at ambient temperature while there was no change in a chloroform-acetone mixture (De Mola et al, 1975). Impurities such as calcium or magnesium ion may act as a catalyst (Kim et al., 2006) and higher temperatures can accelerate the rate of degradation (Ramsay et al., 1994).

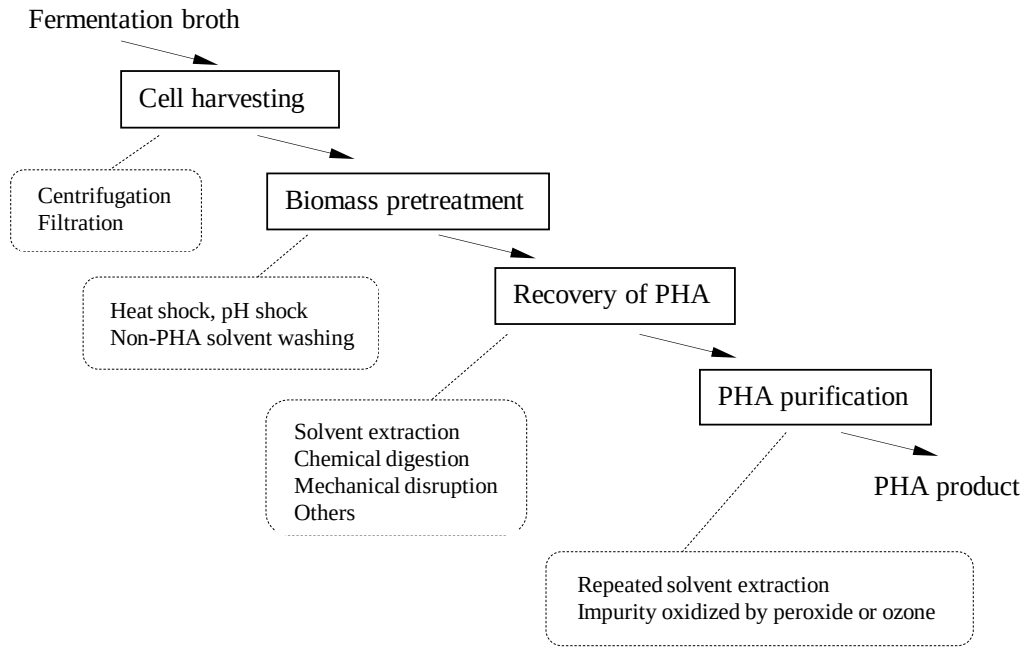

Figure 2-5 Schematic diagram of a general PHA recovery process.

Table 2-5 Examples of solvents tested in PHB recovery

Solvent type	Solvent examples	Reference
Halogenated hydrocarbon	Chloroform, methylene chloride, chloroethane, chloropropane	Baptist, 1962; Coty, 1966 Vanlautem and Gilain, 1982
Cyclic carbonate	Ethylene carbonate, propylene carbonate	Lafferty and Heinzle, 1978
Diol and triol	1,2-propanediol	Traussnig et al, 1990
Solvent mixture	Methylene chloride with ethanol	Baptist, 1962

The U.S. Food and Drug Administration requires that there can only be a maximum of 5.0 endotoxin units (EU) per kilogram body weight per injection. *Escherichia coli* cells (50 g L^{-1} containing 69% PHB) contain more than 10^7 EU per gram PHB (Lee et al., 1999). Using solvents can reduce the endotoxin levels. Less than 10 EU per gram PHB could be achieved when *Ralstonia eutropha*, *Alcaligenes latus* or recombinant *E. coli* biomass was acetone pre-treated and subsequently extracted in chloroform at 30^{o}C for 48 h. Similarly, solubilization of poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) from *P. putida* GPo1 with n-hexane or 2-propanol resulted in an endotoxicity level below 15 EU per gram PHO (Furrer et al., 2007b).

Solvent extraction has been well developed for PHB recovery, but not for MCL-PHA. When MCL-PHA was discovered, SCL-PHA had already entered the early commercialization stage. Therefore, it was not surprising that PHB solvent extraction methods were applied to MCL-PHA (Durner et al., 2001; Sanchez et al., 2003). However, it is known that the solubility of different PHAs differs among solvents (Terada and Marchessault, 1999). For example, most MCL-PHAs are readily soluble in acetone, while SCL-PHAs are not (Ashby et al., 2002). The efficiency of solvents on MCL-PHA recovery has not been tested probably because MCL-PHA has not been commercially available even in small quantities.

2.3.4 Recovery based on digestion of non-PHA-cellular-material (NPCM)

Digestion methods use a combination of enzymes and/or chemical reagents to break down the cell envelope and release the PHA granules into an aqueous environment. The solubilized NPCM consists of lipids, proteins, nucleic acids, peptidoglycan and other carbohydrates from both the cell envelope and the cytoplasmic matrix. Heat treatment

before digestion can denature nucleic acids, some proteins and polysaccharides and can prevent an increase in viscosity when these molecules are released from the cell (Holmes et al., 1990). Some proteins will dissolve upon heating (Cumming and Watson, 1997).

Enzyme digestion is practical on a large-scale and can be used alone or with other reagents. A variety of enzymes may be required, e.g. proteinases, phospholipases, deoxyribonucleases and lysozyme. Enzyme mixtures may give a higher PHA purity compared to a single enzyme or enzymes used sequentially (Holmes et al., 1990). Efficient and cheap enzymes are critical for cost reduction. Under optimized production and enzymatic digestion conditions, MCL-PHA could be produced for a total cost of 5 US$/kg (DeKoning et al., 1997). However, this price is still not competitive with conventional plastics.

Chemical reagents used in digestion include surfactants, preferably ionic surfactants such as sodium dodecyl sulfate (SDS), and chelators such as ethylene diamine tetraacetic acid (EDTA). Table 2-6 shows some of the enzymes and other reagents that have been investigated. Hypochlorite has also been shown to be effective in digestion. Digestion of 1% *Alcaligenes eutrophus* biomass containing 50% PHB in hypochlorite solution (pH 13.6) at 25^{o}C for an hour resulted in 98% PHB, but the molecular weight had decreased to one fifth of its control value of 1,200,000 (Berger et al., 1989). A 50% loss of the molecular weight was still observed under optimized conditions. A combination of hypochlorite digestion and solvent (chloroform) extraction was better and resulted in PHB with a purity of 97% and a molecular weight close to its original value (Hahn et al., 1993). The chloroform extracted the released PHA granules from the hypochlorite phase and protected the polymer from further degradation.

Alkaline treatment had been frequently considered as a pretreatment option that could be combined with other methods. However, it has proven effective in PHB recovery from recombinant *E. coli* (Choi and Lee, 1999). A product containing up to 98% PHB (from a biomass that originally contained 77% PHB) was obtained after 50 g L^{-1} cells were digested in 0.2 N NaOH at 30^{o}C for 1 h. Such a process could potentially reduce the total production cost to 3.66 US$/kg. This method has not been evaluated on other microorganisms or for MCL-PHA recovery.

Table 2-6 Enzymes and reagents used for PHA recovery

Producing strain	Type of PHA	Enzymes and chemical reagents	Product purity	Reference
R. eutropha NCIB 11599	SCL-PHA	Alcalase, lecitase, lysozyme, protease, esparase, neutrase, bromelain, papain, SDS, EDTA, etc.	70-90%	Holmes and Lim, 1990
R. eutropha DSM545	SCL-PHA	Papain, bromelain, bovine chymotrypsin, lysozyme, cellulase, pancreatin	60-90%	Kapritchkoff et al., 2006
P. putida GP01, *P. Putida* KT2442	MCL-PHA	Lysozyme, neutrase, alcalase, lecitase, EDTA, SDS, etc.	92-98%	deKoning and Witholt, 1997

Mechanical cell disruption methods such as the French press, bead milling and ultrasound (Hwang et al., 2006; Ling et al., 1997; Tamer et al., 1998) are frequently used in combination with chemical methods to enhance digestion of the non-PHA cellular components.

2.4 Quantitative analysis of PHA

Quick and reliable quantitative analysis of PHA is important for the development of PHA research and large scale production. Analytical methods have improved since Lemoigne discovered PHB in 1926. He saponified the extracted polymer and determined the PHB content by gravimetry. This required a large amount of biomass to achieve accurate analysis and was time consuming.

Later, methods based on visible (Williamson and Wilkinson, 1958) and ultraviolet (UV) (Law and Slepecky, 1961) spectrophotometry were developed. The UV method uses concentrated sulfuric acid to catalyze the conversion of PHB to crotonic acid followed by quantification at 235 nm. It can analyze as little as 5 μg of PHA. However, spectrophotometric assays cannot differentiate different monomers and therefore cannot be used to determine monomeric composition. Similar drawbacks exist with methods based on Fourier transform infrared spectroscopy (Randriamahefa et al., 2003; Jarute et al., 2004).

The combination of high performance liquid chromatography and UV spectro-photometric made quick analysis possible (Karr et al., 1983) but gas chromatography (GC) (Braunegg et al., 1978) is simpler and more powerful. In the latter method, the polymer's monomers were converted to 3-hydroxyalkanoic acid methyl esters using methanol and 3% sulfuric acid, then separated in a GC column and quantified with a flame ionization detector. Use of an internal standard (usually benzoate) increased accuracy. This method became widely accepted in PHA analysis and has become the most common way to report PHA purity. Later, the GC method was adapted to analyze MCL-PHA using15% sulfuric acid at 100^{o}C for 140 min with good linear results from

0.15 to 15 mg (Lageveen et al., 1988). After studying the effect of time on the formation of the methylesters, Huijberts et al. (1994) suggested extending the heating for 4 h. More recently, transesterification reaction has been proposed using boron trifluoride as a catalyst to avoid the underestimation of MCL-PHA because it is a more efficient reaction especially in the case of functionalized polymers (Furrer et al., 2007a). Although there have been studies to improve this analysis, there are several sources of errors, such as the random variations in methylester preparation. Consequently, it is usual to obtain a variation of about $\pm 3\%$ for the same sample.

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Chapter 3

Biosynthesis and properties of medium-chain-length polyhydroxy-alkanoates with an enriched content of the dominant monomer

This chapter was prepared for submission to Biomacromolecules.

3.1 Abstract

When grown in a nonanoic acid-limited chemostat at a dilution rate of 0.25 h^{-1}, *Pseudomonas putida* KT2440 produced poly(3-hydroxynonanoate-co-3-hydroxyheptanoate) containing 68 mol% 3-hydroxynonanoate (HN) and 32 mol% 3-hydroxyheptanoate (HHp) under unregulated conditions. With acrylic acid, a fatty acid β-oxidation inhibitor, HN monomer content increased to 88 mol%. Co-feeding glucose (3.9 g L^{-1}) and nonanoic acid (2.9 $\pm$ 0.1 g L^{-1}) in continuous culture with increasing amounts of acrylic acid (0 ~ 0.2 g L^{-1}) enriched HN content to 95 mol%. Biomass production was not sacrificed and a yield of PHA from nonanoic acid as high as 0.93 mol mol^{-1} was attained. Poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) with a 3-hydroxyoctanoate (HO) content of up to 98 mol% was similarly produced from octanoic acid. As the dominant monomer content increased, the melting point of the poly(3-hydroxynonanoate) copolymers increased from 46 to 63^{o}C, and that of the poly(3-hydroxyoctanoate) copolymers from 54 to 62^{o}C. All copolymer compositions resulted in elongation to break values of about 1300%. Tensile strength at break, Young's modulus increased with increasing amounts of the dominant monomer.

3.2 Introduction

Poly(3-hydroxyalkanoates) (PHAs) are compostable polyesters synthesized by a variety of microorganisms from renewable resources. Short-chain-length PHA (SCL-

PHA) and short-chain-length-co-medium-chain-length PHA (SCL-MCL-PHA) have been or are planned to be produced on a commercial scale. These materials are poly(3-hydroxy-butyrate) (PHB) or copolymers whose dominant monomer is 3-hydroxybutyrate (HB). PHB homopolymer is a relatively high crystalline material. As a thermoplastic, its major limitations are brittleness, low elongation at break and a melting point (about 180^{o}C) close to its degradation temperature (Holmes, 1985). As a result, PHB has limited commercial applications and is difficult to melt process. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(HB-HV)) and poly(3-hydroxybutyrate-co-3-hexanoate) (P(HB-HHx)) copolymers were developed to alleviate these problems (Holmes, 1988; Green, 2001; Nomura et al., 2008). Side-chains of monomers other than HB generally interfere with crystal formation, producing materials with lower or no melting point and greater elasticity. The melting temperature and crystallinity of P(HB-HV) decreases from 180 to 140^{o}C and from 80 to 30%, respectively, as the mole percentage of 3-hydroxyvalerate (HV) increases from 0 to 25% (Holmes, 1988). Similarly, the melting temperature of P(HB-HHx) copolymers can decrease from 180 to 80^{o}C by increasing the 3-hydroxyhexanoate (HHx) content from 0 to 25 mol% (Green, 2001).

Medium-chain-length PHAs (MCL-PHAs) have six or more carbons in the repeating units. In contrast to SCL-PHAs, MCL-PHAs have much lower crystallinities and crystallization rates, which were attributed to the heterogeneousness of their monomeric compositions (Van der Walle et al., 2001). Almost all naturally occurring MCL-PHAs are copolymers because the 3-hydroxy-carboxylic acid substrates in their biosynthetic pathway are subject to β-oxidation, providing additional lower molecular weight substrates for PHA synthesis. It is therefore desirable to develop strategies to control the

monomeric composition of MCL-PHA in order to obtain materials of desired crystallinity and melting temperature. To this end, genes encoding for key β-oxidation enzymes have been amplified or knocked out. For example, deletion of *fadA* and *fadB* and overexpression of *fadD* and *fadE* genes of the β-oxidation pathway in mutant *Escherichia coli* strains harbouring the *Pseudomonas* sp. 61-3 PHA synthase gene enabled the accumulation of MCL-PHA containing up to 80 mol% 3-hydroxydecanoate from 2 g L^{-1} dodecanoate in Lauria broth medium (Park et al., 2003; Ouyang et al., 2007). This approach produced an MCL-PHA with more of the dominant monomer but it was still not homopolymeric. The limitation to the use of genetically modified strains is that it is not obvious how to obtain a range of controlled monomeric compositions with such organisms.

Chemical inhibitors such as acrylic acid may be used to regulate the β-oxidation pathway (Thijsse, 1964), but this could result in poor growth and no PHA accumulation (Huijberts et al., 1994). Recently, it was demonstrated that *Pseudomonas putida* KT2440 could utilize a carboxylic acid (nonanoic acid) and a carbohydrate (glucose) simultaneously during carbon-limited but nutrient-sufficient exponential growth (Sun et al., 2009). All MCL-PHA was derived entirely from nonanoic acid while glucose served as a supplementary carbon and energy source. The objective of the research presented in this chapter is to evaluate the feasibility of combining the feeding of acrylic acid with co-feeding an alkanoic acid and glucose to obtain MCL-PHA with enriched content of the dominant monomer, which may result in higher crystallinity and melting temperature, without sacrificing cell growth and PHA accumulation.

3.3 Materials and methods

3.3.1 Microorganism and growth medium

Pseudomonas putida KT2440 (ATCC 47054) was maintained in lyophiles and on nutrient agar plates. The medium for inoculum preparation contained per liter: $(NH4)_2SO_4$ 4.70 g, $MgSO_4 \cdot 7\ H_2O$ 0.80 g, $Na_2HPO_4 \cdot 7\ H_2O$ 12.00 g, KH_2PO_4 2.70 g, nutrient broth 1.00 g, glucose 9.00 g. The chemostat feed medium contained per liter: $(NH_4)_2SO_4$ 5.02 g, $MgSO_4 7H_2O$ 1.03 g, $Na_2HPO_4 7H_2O$ 2.24 g, KH_2PO_4 0.50 g, trace element solution 1.4 mL. Glucose and acrylic acid were fed with mineral salt nutrients, while nonanoic acid was fed from a separate reservoir. The trace element solution contained per liter: $FeSO_4 \cdot 7H_2O$ 10.0 g, $CaCl_2 \cdot 2H_2O$ 3.0 g, $ZnSO_4 \cdot 7H_2O$ 2.2 g, $MnSO_4 \cdot 4H_2O$ 0.5 g, H_3BO_3 0.3 g, $CoCl_2 \cdot 6H_2O$ 0.2 g, $Na_2MoO_4 \cdot 2H_2O$ 0.15 g, $NiCl_2 \cdot 6H_2O$ 0.02 g and $CuSO_4 \cdot 5H_2O$ 1.00 g. Before switching to continuous culture, batch mode was conducted with 1.1 L of the above medium and 3.9 g L^{-1} glucose.

3.3.2 Conditions of continuous cultivation

Inocula (100 mL) were prepared in 500 mL shake flasks at 28.0 $\pm$ 1.0^{o}C and 200 rpm for 16 h. All continuous fermentations were performed in a 1.5 L stirred tank bioreactor (Bioflo IIc, New Brunswick) with a working volume of 1.1 L at 28.5 $\pm$ 1.0^{o}C and pH was automatically controlled at 6.85 $\pm$ 0.05 using 2 N potassium hydroxide. The dissolved oxygen (DO) was monitored with an Ingold polarographic electrode and maintained at or above 30% air saturation with an aeration rate of 0.8 vvm and an agitation speed of up to 900 rpm. The dilution rate of all chemostat experiments was 0.25 h^{-1}.

Carbon dioxide (CO_2) content in the exit gas was measured with an infrared CO_2 monitor (Guardian Plus, Topac Inc. Hingham, MA, USA) and the data was acquired by a

LabVIEW 6.1 program (National Instruments) to calculate the CO_2 production rate (CPR, g L^{-1} h^{-1}). For each culture condition, steady state was assumed after the passage of at least 6 L of medium (i.e. five working volumes), and after the DO and CPR plateaued. Reported chemostat data are the average of at least two samples taken three hours apart at each steady state. Biomass was recovered by centrifugation and PHA extracted for characterization.

3.3.3 Effect of acrylic acid with single substrate or co-substrate feeding

3.3.3.1 PHA synthesis using nonanoic acid as the sole substrate

Cells were grown on nonanoic acid as the only carbon substrate with an excess of all other nutrients. By setting the aqueous medium flow rate at 0.28 L h^{-1} and the nonanoic acid flow rate at 1.06 $\pm$ 0.03 g h^{-1}, the inlet nonanoic acid concentration was maintained at 3.8 $\pm$ 0.1 g L^{-1}. The continuous fermentation was first operated without acrylic acid (acrylic acid 0 g L^{-1}). After steady state was achieved and two samples were taken, the inlet acrylic acid concentration was increased and another set of steady state samples were taken. Inlet acrylic acid concentrations of 0.00, 0.02, 0.05, 0.10, 0.20, and 0.40 g L^{-1} were investigated.

3.3.3.2 PHA synthesis using nonanoic acid and glucose as co-substrates

As in the nonanoic acid-only continuous cultivation described above, a set of acrylic acid concentrations including 0.00, 0.02, 0.05, 0.10, 0.15, 0.20, 0.25, 0.35, 0.45 g L^{-1} were studied while nonanoic acid and glucose were fed as co-substrates. The inlet concentrations of glucose and nonanoic acid were 3.9 and 2.9 $\pm$ 0.1 g L^{-1}, respectively.

3.3.4 Effect of substrate ratio under co-substrate feeding

A set of inlet nonanoic acid to glucose ratios (0.5:1, 0.75:1, 1:1, 1.25:1, 1.4:1, 1.7:1, w/w) were studied at a fixed inlet acrylic acid concentration of 0.20 g L^{-1}. The ratio was achieved by varying the inlet nonanoic acid concentration from 1.9 g L^{-1} to 6.6 g L^{-1} while keeping the inlet glucose concentration constant at 3.9 g L^{-1}.

3.3.5 Co-feeding acrylic acid with glucose and heptanoic or octanoic acid

Heptanoic or octanoic acid were co-fed with glucose, at acrylic acid concentrations of 0.00, 0.02, 0.05, 0.10, 0.20, 0.30, 0.40 g L^{-1}. The inlet glucose concentration was maintained at 3.9 g L^{-1}, while the heptanoic acid and octanoic acid concentrations were maintained at 3.6 $\pm$0.2, and 2.8 $\pm$0.1 g L^{-1}, respectively.

3.3.6 Analytical procedures

3.3.6.1 Biomass and nutrient analyses

Duplicate 10 mL samples were centrifuged at 6,000 $\times g$ for 15 min. Supernatants were saved for substrate and nutrient consumption analyses. Biomass pellets were washed with distilled water, recentrifuged, resuspended, frozen, lyophilized and then weighed to obtain the dry biomass concentration. Glucose in all steady-state samples was measured colorimetrically after reaction with an alkaline 4-hydroxybenzoic hydrazide solution (Lever, 1972). Nonanoic acid was methylated in acidified methanol (Ramsay et al., 1991) and analyzed by the same gas chromatograph conditions used for PHA analysis. Phosphate was measured based on the reduction of phosphomolybdate to molybdene blue (Clesceri et al., 1999). Ammonium was determined by the phenol-hypochlorite method (Weatherburn, 1967). Acrylic acid concentration was assayed using a Hewlett-Packard

GC equipped with a Carbowax®-PEG column and a flame ionization detector after acidification with one tenth volume of 2 N hydrochloric acid (Qi et al., 1998).

3.3.6.2 PHA content and composition

PHA content was determined using a modification of the method described by Lageveen et al. (1988). A known amount of lyophilized biomass or MCL-PHA standard was added to 2 mL chloroform and 1 mL methanol containing 15% (v/v) concentrated sulfuric acid with 0.2% (w/v) benzoic acid as an internal standard. The mixtures were placed in a 100^{o}C water bath for 4 h. After cooling, 1 mL distilled water was added to each sample and mixed vigorously on a Fisher vortex mixer for 1 min followed by phase separation overnight. 1 µL of the organic phase was injected into a CP3900 Varian GC with a flame ionization detector. The injection and detector temperatures were 250 and 275^{o}C, respectively. The oven heating profile was: initial 90^{o}C for 0.5 min, 5^{o}C min^{-1} to 95^{o}C, hold for 0.5 min, 30^{o}C min^{-1} to 170^{o}C and hold for 2.5 min. The identification and quantification of the PHA monomers were determined using a MCL-PHA standard whose purity and composition were determined by GC and proton nuclear magnetic resonance performed at room temperature with a Bruker Avance 200 spectrometer using deuterated-chloroform containing 20 mg mL^{-1} PHA.

3.3.6.3 Molecular weight analysis

Lyophilized biomass containing about 50 mg PHA was mixed with 10 mL distilled tetrahydrofuran for 5 h, then filtered through a 0.2 µm nylon membrane. The molecular weight of the extracted polymer was determined by injection of a 250 µL sample into a Waters 2695 Gel Permeation Chromatograph equipped with a Waters 410 differential refractive index detector calibrated using polystyrene standards. The mobile phase passed

through four Styragel columns of pore sizes 100, 500, 10^3, and 10^4 Å at 1 mL min^{-1} and 40^{o}C.

3.3.6.4 PHA extraction

To obtain PHA for thermal and mechanical analyses sufficient biomass was recovered by centrifugation at 5,000 ×*g* for 30 min. The biomass was washed in distilled water, centrifuged then lyophilized. The dry biomass was washed in 20 volumes of methanol for 5 min followed by PHA extraction in 10 volumes of acetone at room temperature for 20 h. The mixture was filtered (Fisherbrand® G6 glass-fiber membrane) and the PHA was precipitated in ice-cold methanol by drop-wise addition of the acetone phase concentrated with a rotary evaporator. All PHA samples were purified by three cycles of acetone solubilization and methanol precipitation.

3.3.6.5 Thermal properties

Glass transition temperature (T_g), melting point (T_m) and heat of fusion (ΔH) were obtained by differential scanning calorimetry (DSC) using a TA Instruments DSC Q100. Purified PHA samples were melted at 70^{o}C and allowed to crystallize at 25^{o}C for 5 days before analysis. A sample of known mass was placed in an aluminum pan and sealed. The first heating scan was -70 to 80^{o}C at a rate of 5^{o}C min^{-1}. T_m was read as the peak value of the endothermic curve. The sample was held at 80^{o}C for 5 min before cooling to -70^{o}C at 10^{o}C min^{-1} then held at -70^{o}C for 5 min and re-heated to 80^{o}C at 5^{o}C min^{-1}. The onset T_g was obtained during the second heating cycle.

Thermogravimetric analysis (TGA) was performed using a Q500 instrument (TA Instrument, USA). Samples of 13 ~ 15 mg were heated from 20 to 900 ℃ at a heating rate of 10 ℃ min^{-1}.

3.3.6.6 Tensile property testing

PHA sheets were prepared by melting the material in a square glass mold 1.3 mm in depth at 10^{o}C above its melting temperature until a flat sheet was formed without visible air bubbles. The melted sample was covered and pressed with an imide sheet at room temperature for a certain period of time, which was recorded as the annealing time, until the sample sheet could be peeled off the glass mold without deformation. Dumbbell shaped specimens were cut from the sample sheet with a Type V die according to ASTM D638. The width of the narrow part of the specimen was about 3.3 mm while the thickness was between 1.3 and 2.7 mm, depending on the thickness of the original sample sheet. Stress-strain (engineering) analysis was performed at a crosshead speed of 100 mm min^{-1} on an Instron 3369 tensile testing instrument (Norwood, MA, USA) at room temperature. Tensile stress at break (σ_b), Young's modulus (E), and elongation at break (ϵ_b) of each sample were recorded.

3.4 Results

3.4.1 Effect of acrylic acid on cell growth and PHA production in nonanoic acid-limited continuous cultivation

P. putida produced a PHA (known as PHN copolymer) containing 3-hydroxynonanoate (HN) and 3-hydroxy-heptanoate (HHp) when nonanoic acid was used as the sole carbon source. Acrylic acid addition significantly increased HN from 68 mol% to a maximum of 88 mol% (Figure 3-1). Although a low concentration of acrylic acid (0.02 g L^{-1}) seemed to enhance PHA accumulation, both the biomass concentration and especially the PHA content decreased at higher acrylic acid concentrations. No PHA was accumulated when the inlet acrylic acid was higher than 0.2 g L^{-1} and residual acrylic

acid was detected in the outflow medium. Inlet acrylic acid concentrations higher than 0.4 g L^{-1} were not studied as a higher inhibitor concentration showed a clear negative effect on cell growth (acrylic acid toxicity test in Appendix A). Nutrients including nitrogen, phosphate and trace elements in the inlet medium were enough to support at least 7 g L^{-1} biomass. Residual ammonium and phosphate concentrations were both 0.75 $\pm$0.25 g L^{-1}. The carbon source (nonanoic acid) was virtually completely consumed (always less than 0.1 g L^{-1}) confirming that cultivation was conducted under carbon-limited conditions.

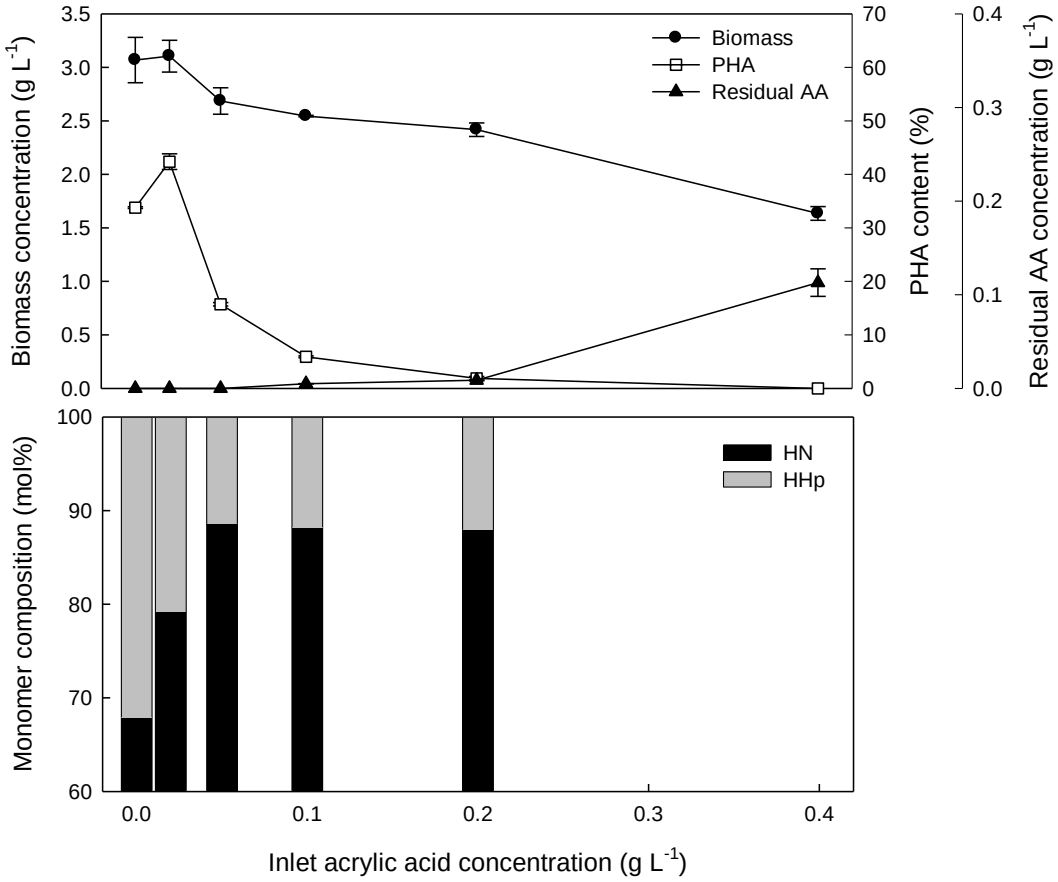


Figure 3-1 Effect of acrylic acid on cell growth and PHA production in nonanoic acid-limited, (3.8 $\pm$0.1 g L^{-1} inlet concentration) continuous culture at D = 0.25 h^{-1}. HN, 3-hydroxynonanoate; HHp, 3-hydroxyhepatanoate; AA, acrylic acid. Symbols are at the average of two steady state samples and error bars represent the range of the average.

3.4.2 Effect of acrylic acid on cell growth and PHA production in carbon-limited, continuous culture with nonanoic acid and glucose co-feeding

In contrast to growth on nonanoic acid alone, when the culture was grown in a chemostat on nonanoic acid and glucose as co-substrates, the biomass concentration was almost unaffected with an inlet acrylic acid concentration of up to 0.45 g L^{-1} (Figure 3-2). PHA content increased dramatically from 11 to 64% when acrylic acid was increased from 0.00 to 0.15 g L^{-1}, accompanied by a substantial increase in HN content from 69 to 95 mol%. Acrylic acid inlet concentrations above 0.15 g L^{-1} caused a small decrease in both PHA content and the HN proportion.

The yield of PHA from nonanoic acid ($Y_{PHA/NA}$) was 0.16 mol mol^{-1} (0.15 g g^{-1}) when no acrylic acid was in the feed, but increased substantially as the inlet acrylic acid concentration was increased. The highest PHA yield from nonanoic acid was 0.92 mol mol^{-1} (0.90 g g^{-1}), 92% of the theoretical maximum, at 0.15 g L^{-1} acrylic acid. Residual acrylic acid built up gradually in the fermentor as the acrylic acid inlet concentration increased.

3.4.3 Effect of nonanoic acid to glucose ratio on PHA content and composition

Increasing amounts of nonanoic acid was provided at a constant acrylic acid concentration by increasing the ratio of nonanoic acid to glucose in the feed. PHA with high HN contents were obtained at low nonanoic acid to glucose ratios (e.g. 98mol% HN at 0.5: 1, Figure 3-3). As the ratio was increased, more biomass was obtained with dramatically lower PHA content and HN molar proportion at a nonanoic acid to glucose ratio above 1.3:1. The PHA yield from nonanoic acid decreased from 0.93 to 0.35 mol mol^{-1} when the nonanoic acid to glucose ratio was increased from 0.5:1 to 1.7:1.

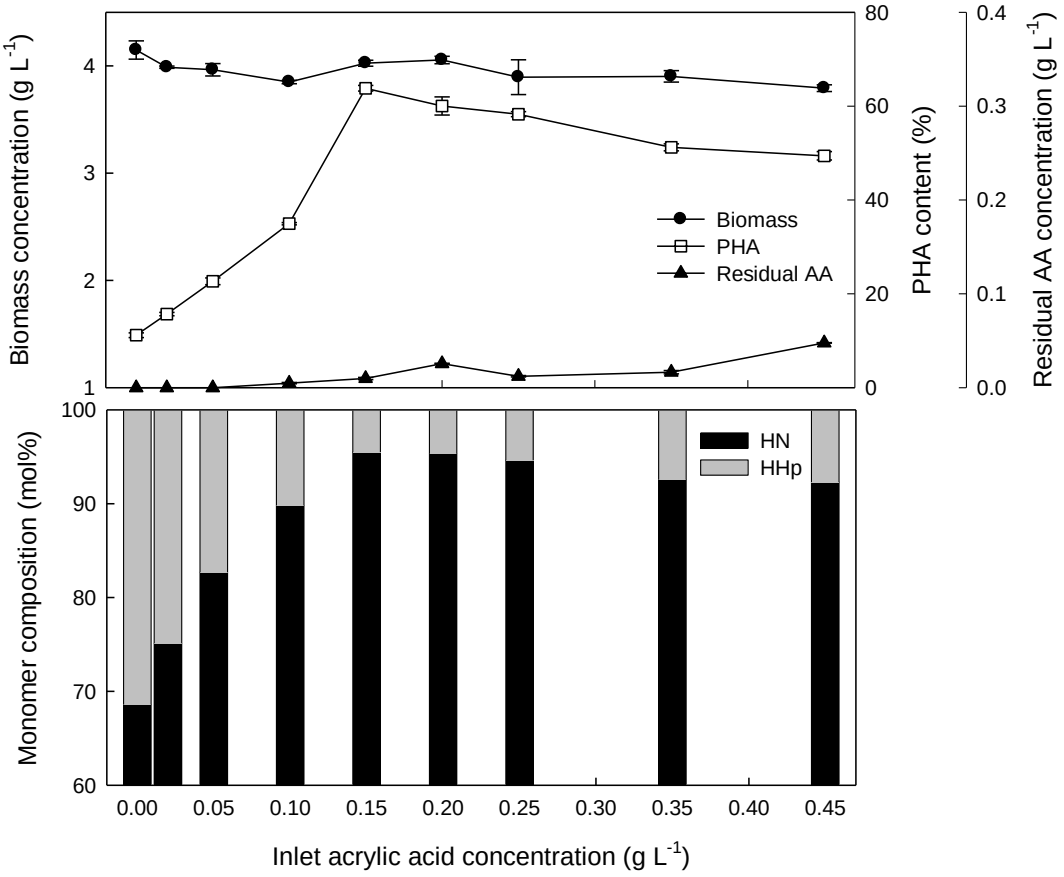


Figure 3-2 Effect of inlet acrylic acid concentration on cell growth and PHA production in continuous cultivation with nonanoic acid (2.9 ± 0.1 g L^{-1} inlet concentration) and glucose (3.9 g L^{-1} inlet concentration) co-feeding at D = 0.25 h^{-1}. The mass ratio of nonanoic acid and glucose in the feed was 0.74:1. HN, 3-hydroxynonanoate; HHp, 3-hydroxy-heptanoate; AA, acrylic acid. Symbols are at the average of two steady state samples and error bars represent the range of the average.

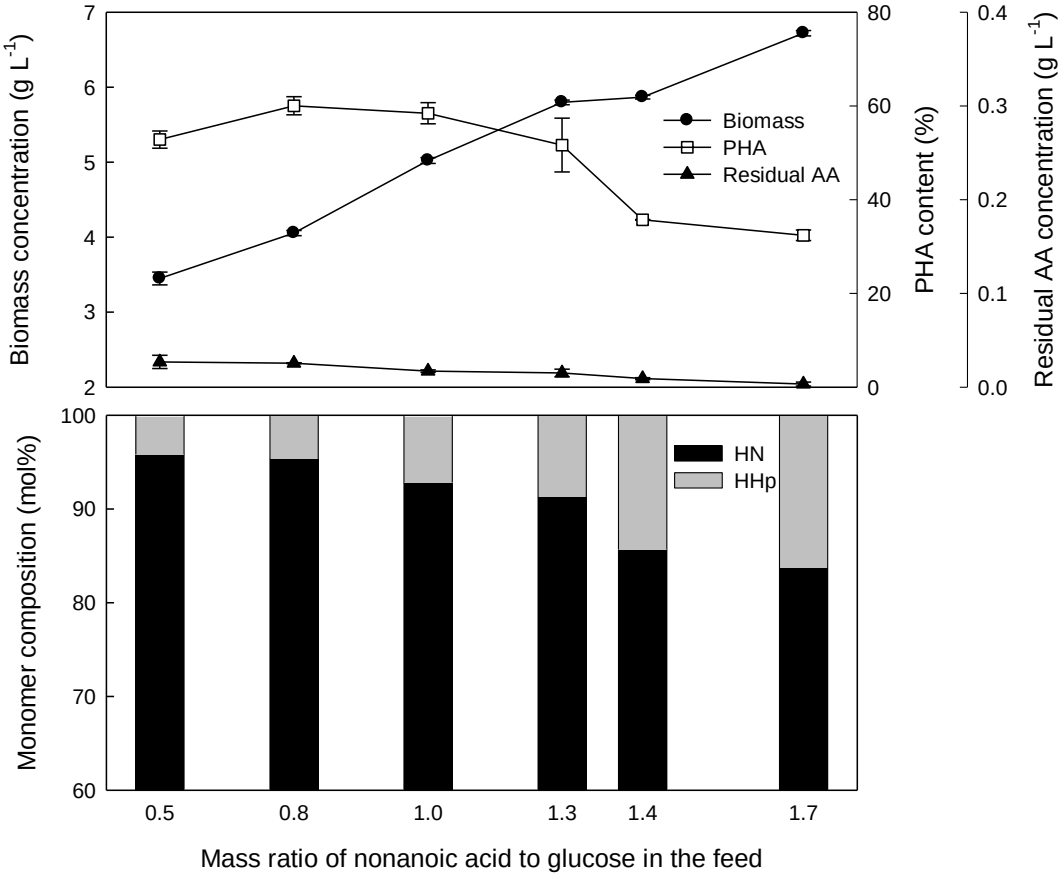


Figure 3-3 Effect of nonanoic acid to glucose feeding ratios on cell growth and PHA production at inlet acrylic acid concentration of 0.2 g L^{-1} in continuous cultivation at D = 0.25 h^{-1}. The inlet glucose concentration was fixed at (3.9 g L^{-1}). HN, 3-hydroxynonanoate; HHp, 3-hydroxyheptanoate; AA, acrylic acid. Symbols are at the average of two steady state samples and error bars represent the range of the average.

3.4.4 Continuous cultivation with acrylic acid and glucose plus octanoic or heptanoic acid co-feeding

P. putida KT2440 grown on octanoic acid and glucose produced similar results as when grown on nonanoic acid and glucose, except that the biomass concentration decreased to a greater extent at higher inlet acrylic acid concentrations (Figure 3-4). Without a β-oxidation inhibitor, the poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) (known as PHO) copolymer contained 88 mol% 3-hydroxyoctanoate (HO). Acrylic acid addition increased the HO fraction, achieving 98 mol% at an inlet acrylic acid concentration of 0.2 g L^{-1}. The PHO content of the biomass was also affected, achieving a maximum of 48%. The PHA yield from octanoic acid ($Y_{PHA/OA}$) was a maximum of 0.67 mol mol^{-1} (0.65 g g^{-1}).

Heptanoic acid was also co-fed with glucose and increasing amounts of acrylic acid. Under these conditions *P. putida* produced a poly(3-hydroxyheptanoate) (PHHp) homopolymer, regardless of the inhibitor concentration. However, the PHHp content in the biomass was less than 3.5%, and its yield from heptanoic acid was lower than 0.05 mol mol^{-1}.

Both PHN and PHO obtained in the continuous fermentations in this study had a weight average molecular weight (Mw) of 150 ± 10 kDa and a polydispersity of 1.55 ± 0.02, regardless of the monomeric composition.

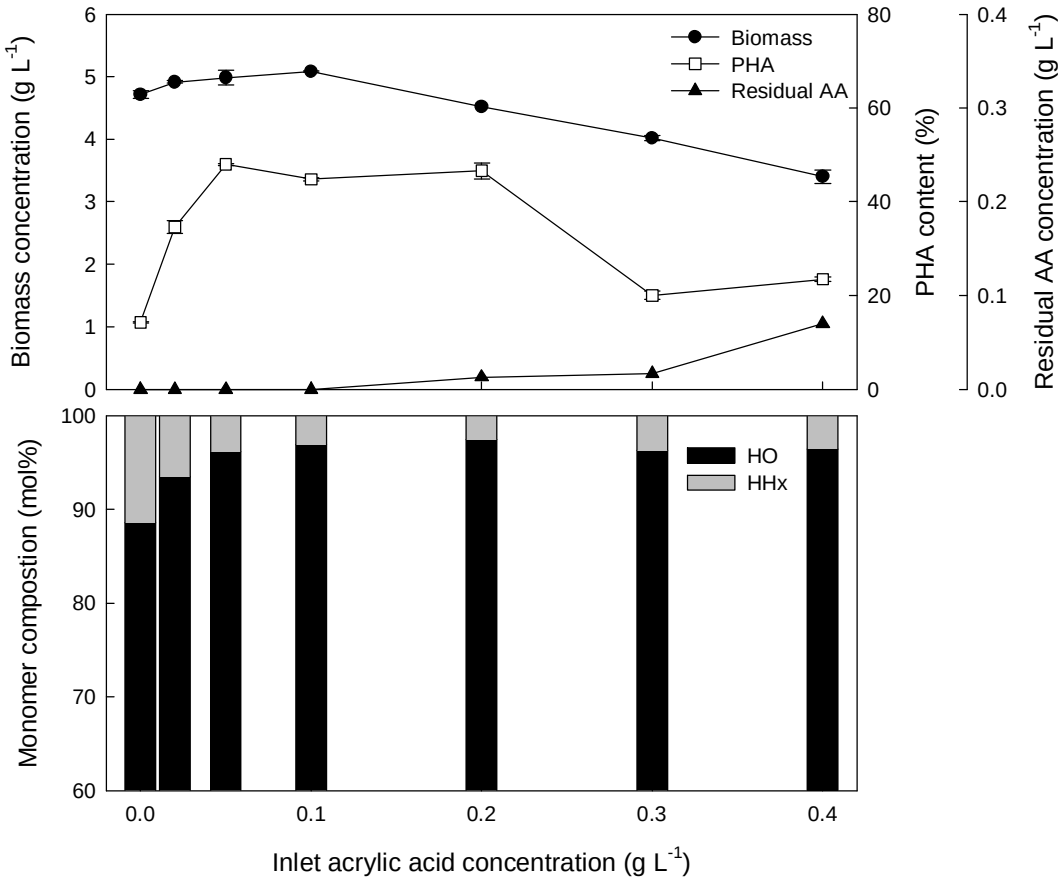


Figure 3-4 Effect of inlet acrylic acid concentration on cell growth and PHA production in continuous cultivation with octanoic acid (3.6 ± 0.2 g L^{-1} inlet concentration) and glucose (3.9 g L^{-1} inlet concentration) co-feeding at D = 0.25 h^{-1}. The mass ratio of octanoic acid and glucose in the feed was 0.92:1. HO, 3-hydroxyoctanoate; HHx, 3-hydroxyhexanoate; AA, acrylic acid. Symbols are at the average of two steady state samples and error bars represent the range of the average.

3.4.5 Thermal properties

The peak melting point of both PHN and PHO copolymers was directly related to the amount of the dominant monomer (Figure 3-5 and 3-6 a, b). For example, as the HN content of PHN increased from 70 to 95 mol% (PHN-95), the peak melting temperature (T_m) increased from 46 to 63^oC. Similarly, when the HO content in PHO increased from 88 to 98 mol%, T_m increased from 54 to 62^oC. Based on an extrapolation of the data (Figure 3-5), a PHN homopolymer would have a T_m of 67^oC while a PHO homopolymer should have a T_m of 64^oC.

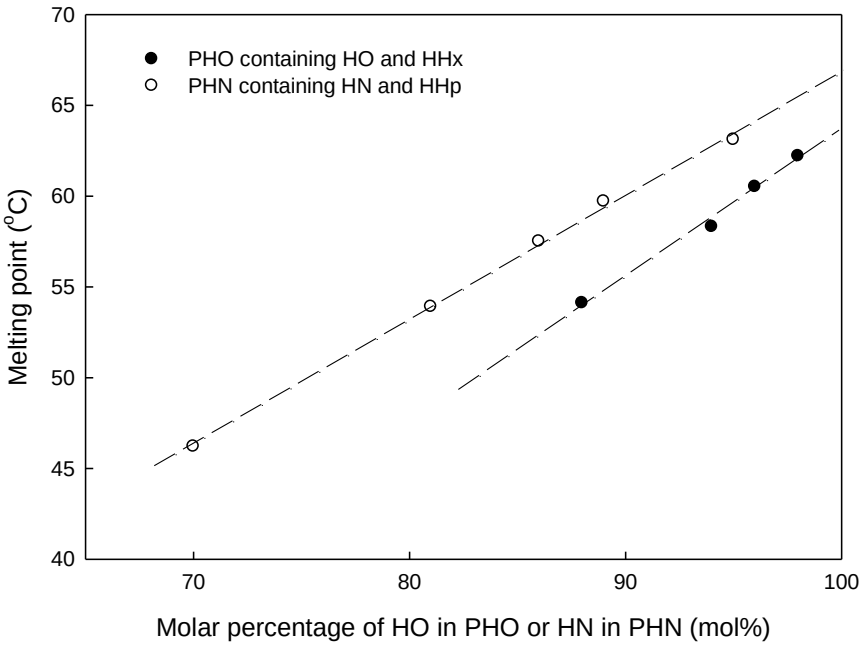
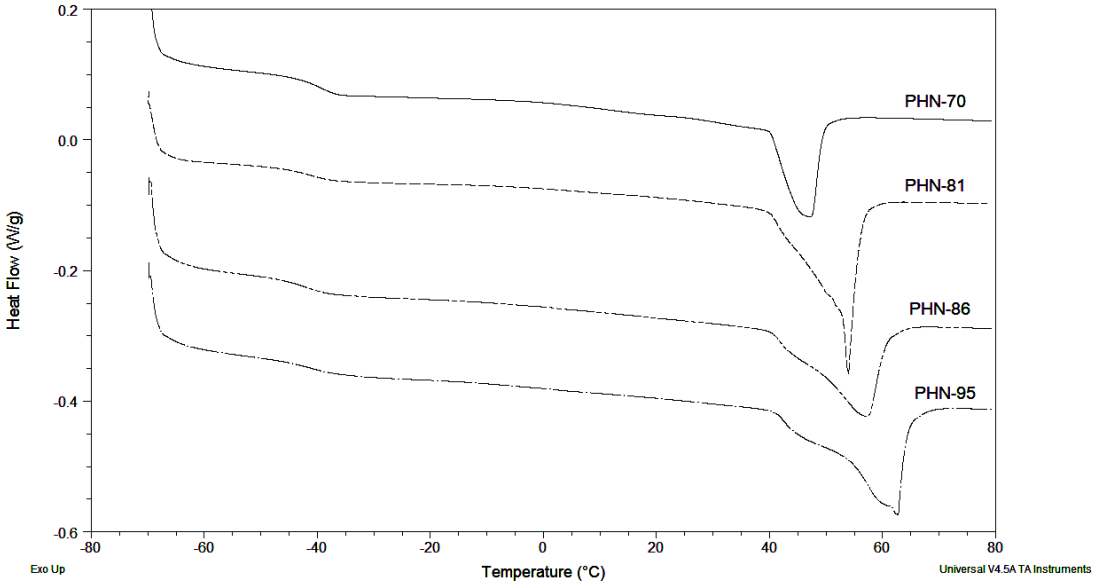
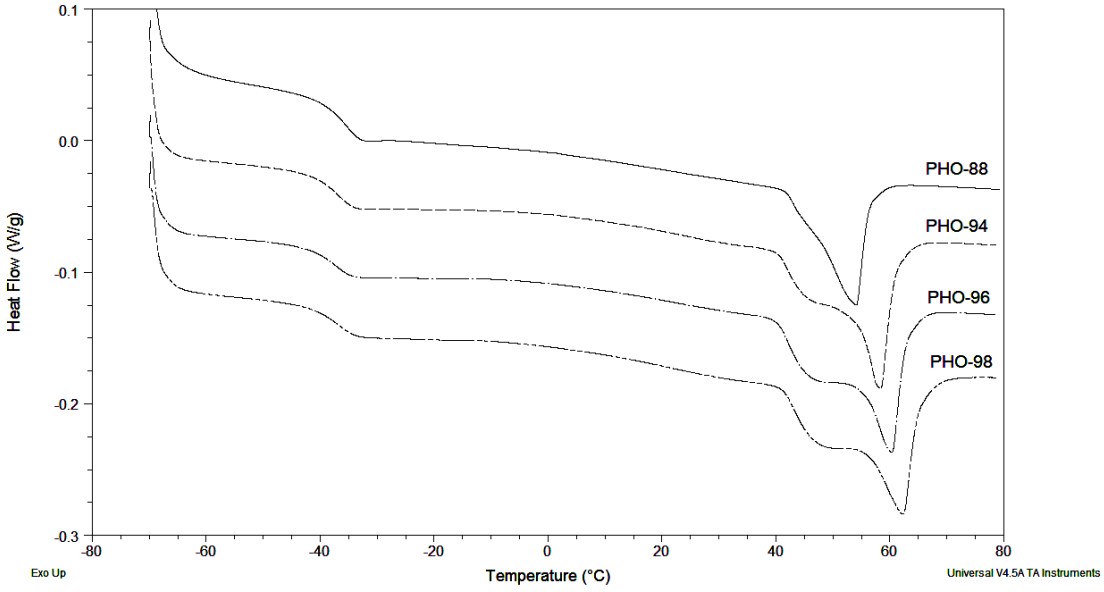


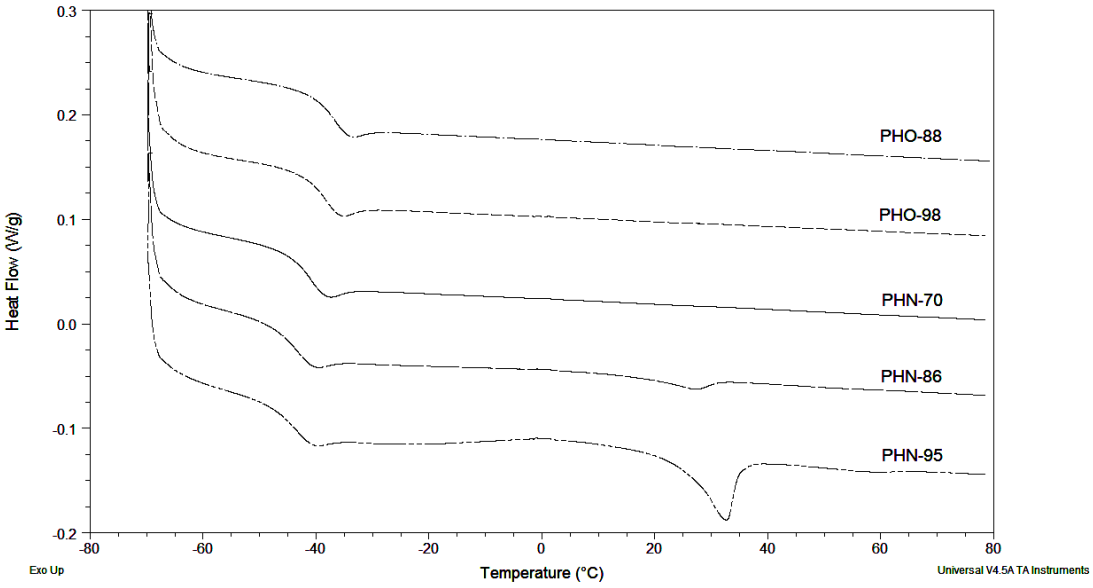
Figure 3-5 Relationship of the melting point of PHO and PHN and the amount of the dominant monomer. HO, 3-hydroxyoctanoate; HHx, 3-hydroxyhexanoate; HN, 3-hydroxynonanoate; HHp, 3-hydroxyhepanoate.


0.2
0.0
-0.2
-0.4
-0.6
Heat Flow (W/g)
PHN-70
PHN-81
PHN-86
PHN-95
-80
-60
-40
-20
0
20
40
60
80
Exo Up
Temperature (°C)
Universal V4.5A TA Instruments

(a)


0.1
0.0
-0.1
-0.2
-0.3
Heat Flow (W/g)
PHO-88
PHO-94
PHO-96
PHO-98
-80
-60
-40
-20
0
20
40
60
80
Exo Up
Temperature (°C)
Universal V4.5A TA Instruments

(b)



(c)

Figure 3-6 Overlay of DSC thermographs of various MCL-PHA produced by *P. putida* KT2440. (a) The first heating scan of PHN containing 70, 81, 86, 95 mol% HN (shown as PHN-70, PHN-81, PHN-86, PHN-95); (b) the first heating scan of PHO containing 88, 94, 96, 98 mol% HO (shown as PHO-88, PHO-94, PHO-96, PHO-98); (c) the second heating scan of PHO-88, PHO-98, PHN-70, PHN-86 and PHN-95.

The glass transition temperature (T_g) of all PHO copolymers was slightly higher than those of the PHN copolymers (Figure 3-6(c)). During the second heating scan, only the PHN samples containing 86 mol% HN (PHN-86) and 95 mol% HN (PHN-95) demonstrated any crystallinity with PHN-95 having the larger melting peak. Based on the heat of fusion PHN copolymers with the same dominant monomer content as the PHO copolymers were more crystalline (Table 3-1).

Thermogravimetric analyses (Figure 3-7) showed that the thermal decomposition temperature of the PHN samples was above 230^{o}C, much higher than the T_m. Increasing the HN from 70 to 95 mol% increased the material decomposition temperature. Similar results were obtained with the PHO copolymers.

Table 3-1 Thermal properties of PHN and PHO with different monomeric compositions

	HN or HO (mol%)	Glass transition temperature T_g (°C)	Melting point (peak) T_m (°C)	Melting point (range) T_m (°C)	Heat of fusion ΔH (J g^{-1})
PHN	70	-45	46	40-52	12
	81	—*	54	40-60	19
	86	-47	58	40-65**	20
	89	—*	60	40-68**	22
	95	-48	63	40-70**	27
PHO	88	-40	54	41-60	9
	94	—*	58	40-65**	12
	96	—*	61	40-66**	14
	98	-42	62	40-71**	15

*Samples not analyzed. **Bimodal distribution.

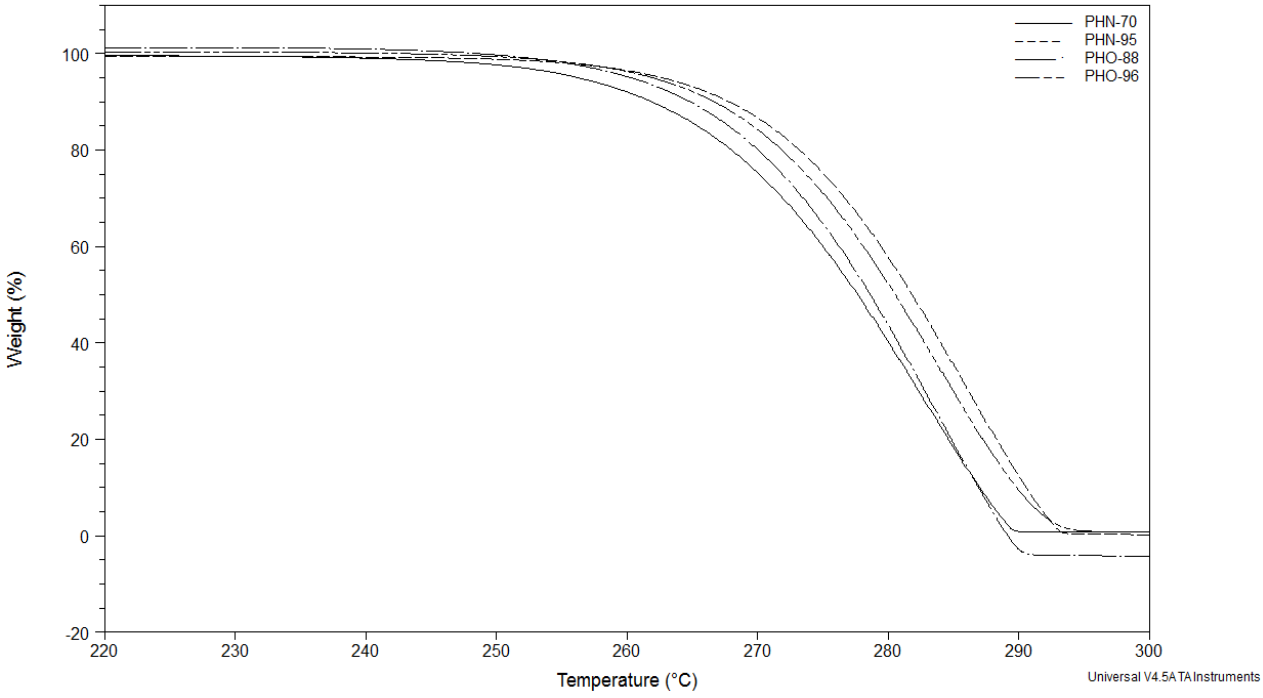


Figure 3-7 Thermogravimetric analysis thermographs of PHN containing 70 mol% HN (PHN-70) and 95 mol% HN (PHN-95) and that of PHO containing 88 mol% HO (PHO-88) and 96 mol% HO (PHO-96).

3.4.6 Tensile properties

The PHN and PHO samples had high values of elongation at break (Table 3-2), and the values were affected very little by their monomeric composition. In contrast, the tensile stress at break and Young's modulus of both PHN and PHO increased significantly with increasing amount of the dominant monomer. The PHN-95 tensile stress at break and Young's modulus values showed two and eight fold increases, respectively, when compared to PHN-70.

3.5 Discussion

3.5.1 Metabolic regulation to control MCL-PHA content and composition in *P. putida* KT2440

Fatty acid β-oxidation is the central metabolic pathway when *Pseudomonas* species are grown on fatty acids (Fiedler et al., 2002). In each cycle of β-oxidation (Figure 3-8 (b)), not only are intermediates generated as MCL-PHA precursors (Figure 3-8 (a)), but acetyl-CoA which is also formed may feed into the tricarboxylic acid cycle (Figure 3-8 (c)). Hence, MCL-PHAs synthesized from fatty acids are copolymers with monomeric structures closely related to the structure of the original carbon substrates. For example, MCL-PHA synthesized from nonanoic acid as the sole carbon source has about 68 mol% HN and 32 mol% HHp. This study used acrylic acid, a β-oxidation inhibitor which interacts with acyl-CoA synthetase (which catalyzes the reaction from the fatty acid to acyl-CoA, Figure 3-8 (b)) and with ketoacyl-CoA thiolase (which catalyzes the reaction from ketoacyl-CoA to acyl-CoA and acetyl-CoA, Figure 3-8 (b)) (Thijsse, 1964), to regulate fatty acid β-oxidation and achieve control of the monomeric composition during MCL-PHA synthesis.

Table 3-2 Tensile properties of PHN and PHO with increasing amounts of the dominant monomer

HN or HO (mol%)	Annealing time at room temperature (h)	Sample thickness (mm)	Tensile stress at break σ_b (MPa)	Young's modulus E (MPa)	Elongation at break ϵ_b (%)
PHN					
70	96	2.64 ± 0.02	6.6 ± 0.6	1.5 ± 0.1	1327 ± 37
85	60	1.87 ± 0.02	10.3 ± 0.7	5.4 ± 0.6	1222 ± 70
90	18	1.56 ± 0.35	12.0 ± 0.5	5.8 ± 0.7	1232 ± 25
95	18	1.47 ± 0.04	15.7 ± 1.0	11.3 ± 1.4	1317 ± 86
PHO					
88	66	1.29 ± 0.04	9.6 ± 0.1	3.4 ± 0.2	1384 ± 46
94	66	1.35 ± 0.02	11.7 ± 0.7	4.7 ± 0.2	1267 ± 91

Error is reported as standard deviation of triplicate samples.

In the production of the PHN copolymer, acrylic acid inhibition of β-oxidation of nonanoic acid increased the HN content, and had negative effect on both cell growth and PHA accumulation during nonanoic acid-limited growth (Figure 3-1). Since this effect was likely due to decreased flux of acetate to the tricarboxylic acid cycle, glucose was provided as another source of acetate (via the Entner-Doudoroff pathway) as in Figure 3-8 (c). *P. putida* KT2440 can produce MCL-PHA when grown on glucose (Sun et al., 2007a), but it has already been demonstrated that this did not occur under carbon-limited conditions even when two carbon sources were used (Sun et al., 2009). In this study, good cell growth was achieved while the amount of PHA accumulated and its HN content depended on the amount of acrylic acid fed (Figure 3-2 and Figure 3-4).

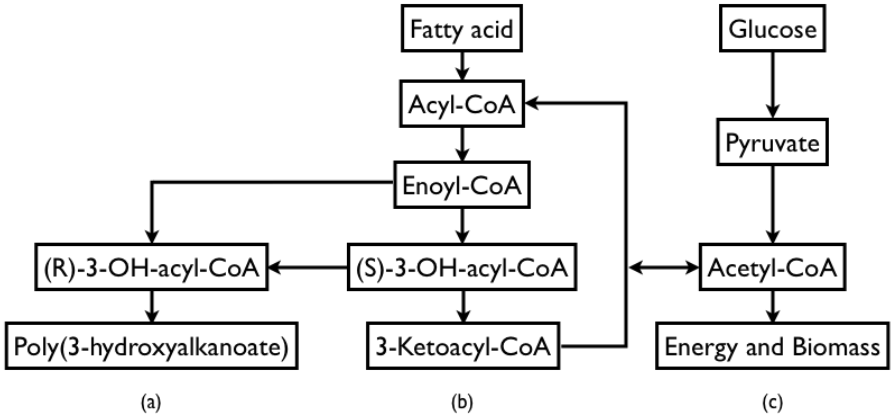


Figure 3-8 Major metabolic pathways in *P. putida* KT2440 when grown on fatty acid and glucose as co-substrates. (a) PHA synthesis, (b) fatty acid β-oxidation, and (c) glucose utilization (Adapted from Huijberts et al., 1994; Vanderleij and Witholt, 1995)

The co-feeding strategy with a β-oxidation inhibitor also allowed an increased HO content in the PHO copolymer indicating that it may be possible to apply this approach to the production of other types of PHA synthesized from fatty acids with longer chain lengths to obtain unique monomeric compositions at a high efficiency. Although an

increase in the dominant monomer content has been achieved using genetically modified organisms (Park et al., 2003; Ouyang et al., 2007), flexible control of the monomeric composition has never been previously achieved.

Moreover, higher yields of PHA from nonanoic acid (up to 0.93 mol mol^{-1} (0.91 g g^{-1})) and from octanoic acid (up to 0.67 mol mol^{-1} (0.65 g g^{-1})) were obtained compared to literature values (0.60 g g^{-1} for $Y_{PHA/NA}$, (Sun et al., 2007b); 0.40 ~ 0.63 g g^{-1} for $Y_{PHA/OA}$ (Kim et al., 1997; Hazenberg and Witholt, 1997)).

However, there was always some co-monomer in the polymer. The dominant monomer of PHN or PHO achieved a maximum (95 and 98 mol%, respectively) then declined as the acrylic acid concentration was increased in the feed (Figure 3-2 and Figure 3-4). Metabolic analysis of the PHA and biomass data (developed by Bozhi Sun and presented in Appendix B) was based on the equilibria of the flux of the intermediates and assumed similar biosynthetic reactions to *E. coli* (Varma and Palsson, 1993). This study revealed a decreased flux of acetate entering the tricarboxylic acid cycle from β-oxidation, followed by a gradual increase indicating a reactivation of the β-oxidation pathway. It is likely that the culture possessed more than one set of enzymes that are involved in β-oxidation where one set was induced upon inhibition of the other as previously shown in *Pseudomonas putida* U (Olivera et al., 2001).

3.5.2 Monomer content-dependent material properties

Since its discovery, MCL-PHA has been synthesized using a variety of strains (mainly *Pseudomonas*) and carbon sources (such as alkanes, alkanoates, vegetable oils, animal fats, and carbohydrates) (Lageveen et al., 1988; Diard et al., 2002). Because the synthetic enzymes have relatively low substrate specificity for MCL precursors, the polymer compositions are usually very complex. In addition, the reported thermal and

mechanical properties of these materials vary significantly possibly due to different recovery and characterization procedures (Van der Walle et al., 2001). This makes it difficult to develop relationships between the materials' thermal and mechanical properties and their monomeric composition. In this study, a range of MCL-PHA polymers with different monomeric composition but similar molecular weight (150 ± 10 kDa) were produced. Identical extraction and sample preparation methods were used for thermal and mechanical testing.

As the proportion of the dominant monomer increased in either PHN or PHO polymers (Figure 3-5and Table 3-1), their T_g decreased and T_m increased due to a decrease in the side chain mobility as they became more like homopolymers. When produced without acrylic acid, PHO (88 mol% HO) had a higher T_m (54^oC) than PHN (70 mol% HN, T_m 46^oC) mainly because PHO had more of the dominant monomer. A linear relationship between the peak T_m and the dominant monomer content of either PHN or PHO was generated (Figure 3-6). The T_m of their corresponding homopolymers was extrapolated to 67^oC and 64^oC, respectively, which agrees with those predicted by other researchers (Gross et al., 1989; Preusting et al., 1990). The relative degree of crystallization, determined from the heat of fusion (ΔH), increased steadily (from 12 J g^{-1} to 27 J g^{-1} for PHN and from 9 J g^{-1} to 15 J g^{-1} for PHO) with increasing dominant monomer content. When the proportion of the dominant monomer of both PHN and PHO increased, the polymers also showed slightly higher thermal stability in terms of the thermal decomposition temperature.

Based on a general comparison of PHN (95 mol% HN) and PHO (98 mol% HO) in Figure 3-6 and Table 3-1, it can be postulated that the PHN homopolymer would have a

higher melting point, rate of crystallization, and degree of crystallinity than the PHO homopolymer, which can probably be attributed to greater involvement of the side-chains in crystallization.

Previous tensile studies had to compromise on the sample dimensions (i.e. width 4 mm, thickness 0.1 mm, (Ouyang et al., 2007) probably due to a lack of polymer availability. Using ASTM D638 standard methods, all samples in this study had much higher elongation at break (about 1300%) than previous data (usually 100 to 450%) (Van de Walle et al., 2001; Ouyang et al., 2007; Liu and Chen, 2007) and the values were independent of the range of the dominant monomer tested.

Other mechanical properties were significantly improved when the amount of the dominant monomer was maximized. The rate of crystallization was affected as it was much easier to obtain a non-sticky sample sheet with PHN-95 than with PHN-70 as seen by the shorter annealing time in Table 3-2. Both higher tensile stress at break and Young's modulus were achieved by increasing the proportion of the dominant monomer. Compared to PHO, the longer side-chains of PHN resulted in both higher tensile stress at break and higher Young's modulus (Table 3-2).

3.1 Conclusions

Metabolic regulation using a combination of (i) fatty acid and carbohydrate substrates and (ii) a β-oxidation inhibitor allowed control of the monomer content in the production of MCL-PHA with 68 to 96 mol% HN or 88 to 98 mol% HO being achieved. A high yield of PHA from fatty acids (i.e. $Y_{PHN/NA}$ 0.93 mol mol^{-1}) under β-oxidation inhibition was obtained only with carbohydrate co-feeding. This could also result in

significantly lower production costs since carbohydrates are much less expensive than fatty acids.

More desirable properties were achieved by increasing the amount of the dominant monomer. Higher dominant monomer content promoted crystallinity and resulted in improved thermal and mechanical properties. An increase of T_m from 46 to 63 ^oC for PHN copolymers or 54 to 62^oC for PHO copolymers was observed. Tensile stress at break and Young's modulus also increased.

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Chapter 4

Fed-batch production of MCL-PHA with elevated 3-hydroxynonanoate content

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4.1 Abstract

With no inhibition of β-oxidation, *Pseudomonas putida* KT2440 produced medium-chain-length poly(3-hydroxyalkanoate) (MCL-PHA) with approximately 65 mol% 3-hydroxynonanoate (HN) from nonanoic acid. Production of PHA with higher HN content and an adjustable monomeric composition was obtained using acrylic acid, a fatty acid β-oxidation inhibitor, together with nonanoic acid and glucose as co-substrates in fed-batch fermentations. Different monomeric compositions were obtained by varying the feeding conditions to impose different specific growth rates and inhibitor feed concentrations. At a nonanoic acid, glucose and acrylic acid feed ratio of 1.25: 1: 0.05 and a specific growth rate of 0.15 h^{-1}, 71.4 g L^{-1} biomass was produced containing 75.5% PHA with as much as 89 mol% HN at a cumulative PHA productivity of 1.8 g L^{-1} h^{-1}.

4.2 Introduction

Poly(3-hydroxyalkanoates) (PHAs) are a family of biodegradable, and non-cytotoxic biopolyesters produced from renewable resources. Certain types of PHAs, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(HB-HV)) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(HB-HHx)), have been recognized as substitutes for petroleum-based thermoplastics in various applications and have been or are planned to be produced commercially (Poirier et al., 1995; Philip et al., 2007).

In contrast to short-chain-length PHAs (SCL-PHAs) such as P(HB-HV) and SCL-MCL-PHAs such as P(HB-HHx), medium-chain-length PHAs (MCL-PHAs) are thermoplastic elastomers with a much higher elongation-to-break (Van der Walle et al, 2001). They also have lower melting temperatures, are less crystalline and crystallize more slowly (Gross et al., 1989; Marchessault et al., 1990; Gagnon et al., 1992). Most bioreactor scale production of MCL-PHAs have used structurally related MCL carbon substrates, such as octane (Hazenberg and Witholt, 1997), nonanoic acid (Sun et al., 2007), and oleic acid (Lee et al., 2000). We have recently demonstrated that using a combination of a PHA structurally related substrate (e.g. nonanoic or octanoic acid), a structurally unrelated substrate (e.g. glucose), and a fatty acid β-oxidation inhibitor (e.g. acrylic acid), a series of poly(3-hydroxynonanoate-co-3-hydroxyheptanoate) (PHN) with adjustable 3-hydroxynonanoate (HN) content (69 to 96 mol%) or poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) (PHO) with adjustable 3-hydroxyoctanoate (HO) content (88 to 98 mol%) can be produced (Chapter 3). It was also demonstrated that the PHA thermal and mechanical properties improved as the amount of the dominant monomer increased (Chapter 3). Substrate utilization efficiency also improved with a yield of fatty acid to PHA conversion as high as 0.91 g g^{-1}. These results were produced in chemostat but, for a variety of reasons, this cultivation technique is not used commercially. In order to be of commercial interest, this novel approach to MCL-PHA production must be shown to be applicable to fed-batch culture.

The objective of this study was to develop a methodology for controlling the monomeric composition of MCL-PHA in efficient fed-batch fermentations. Specifically, the production of PHN copolymers with different HN content was investigated by

controlling the specific growth rate and the β-oxidation inhibitor concentration in the feed. The study also employed nonanoic acid and glucose co-feeding to meet the requirements of both cell growth and PHA accumulation.

4.3 Materials and methods

4.3.1 Microorganism and growth medium

Pseudomonas putida KT2440 (ATCC 47054) was maintained on nutrient agar plates at 4°C. The inoculum medium for all fermentations contained per liter: $(NH4)_2SO_4$ 4.70 g, $MgSO_4 \cdot 7H_2O$ 0.80 g, $Na_2HPO_4 \cdot 7H_2O$ 12.00 g, KH_2PO_4 2.70 g, nutrient broth 1.00 g, glucose 9.00 g. The initial culture medium contained per liter: $(NH_4)_2SO_4$ 4.70 g, $MgSO_4 \cdot 7H_2O$ 0.80 g, $Na_2HPO_4 \cdot 7H_2O$ 18.0 g, KH_2PO_4 4.05 g, trace element solution 10 mL. The trace element solution contained per liter: $FeSO_4 \cdot 7H_2O$ 10.0 g, $CaCl_2 \cdot 2H_2O$ 3.0 g, $ZnSO_4 \cdot 7H_2O$ 2.2 g, $MnSO_4 \cdot 4H_2O$ 0.5 g, H_3BO_3 0.3 g, $CoCl_2 \cdot 6H_2O$ 0.2 g, $Na_2MoO_4 \cdot 2H_2O$ 0.15 g, $NiCl_2 \cdot 6H_2O$ 0.02 g and $CuSO_4 \cdot 5H_2O$ 1.00 g. Nonanoic acid (98%, Spectrum Chemicals) was fed separately in its pure form as it is immiscible in aqueous media. Acrylic acid (Sigma-Aldrich) was added to a glucose (99.5%, Sigma-Aldrich) solution of 240 g L^{-1}. Feeding ratios of nonanoic acid (NA), glucose (G) and acrylic acid (AA) at 1.25: 1: 0.01, and 1.25: 1:0.05 (w/w) were tested. Nitrogen was provided as 14% (w/v) ammonia solution and also served as the base for pH control. To avoid precipitation in the fermentation medium, $MgSO_4 \cdot 7H_2O$ was added to the glucose medium and fed at 0.06 g $MgSO_4 \cdot 7H_2O$ per gram glucose assuming a $Y_{X/Mg}$ of 240 g g^{-1} (Sun et al., 2006). In case of nutrient depletion, supplemental solutions of trace elements with the above composition and a phosphate solution containing 36 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$

and 8.1 g L^{-1} KH_2PO_4 were prepared. Antifoam 204 (Sigma-Aldrich) was added to nonanoic acid (1% v/v) and manually injected through a sterile septum when required.

4.3.2 Fermentation conditions

The inoculum was grown in three 500 mL shake flasks (100 mL medium in each flask) at 28.0 ± 1 ^oC and 200 rpm overnight. The first two fermentations were conducted in a 7 L MBR stirred tank bioreactor (Bioreactor-AG, Switzerland) with a 5 L working volume. The third fermentation was done in a 5 L Minifors bioreactor (Infors-HT, Bottmingen, Switzerland) with a 3 L working volume. The cultivation temperature was 28.5 ± 1 ^{o} C and the pH was controlled at 6.85 ± 0.05 using 14% (w/v) ammonia solution. Dissolved oxygen was measured with an Ingold polarographic probe and maintained above 30% air saturation by adjusting the agitation speed and the mixture of air and oxygen flow via mass flow controllers to a total gas flow at 1 vvm. The dissolved oxygen data were acquired by a LabVIEW 6.1 (National Instrument) program. Nonanoic acid and glucose feeding was controlled via separate peristaltic pumps by the LabVIEW program based on the mass of each reservoir.

4.3.3 Substrate feeding and control methods

The specific growth rate was controlled at 0.25 h^{-1} or 0.15 h^{-1} by exponentially feeding the carbon sources to be the growth-limiting nutrient. It was estimated that 1 g L^{-1} biomass would be produced from 1.6 g L^{-1} total carbon sources in the initial fermentation medium. The cumulative mass of carbon substrates S_t (g) to be fed at time t (h) was calculated based on exponential cell growth (X_t, g) expressed in the equation below.

$$S_t = \frac{X_t}{Y_{X/C}} = \frac{X_0}{Y_{X/C}} \cdot (e^{\mu t} - 1) \quad (1)$$

where X_0 (g) is the estimated biomass at the beginning of the feeding; μ (h^{-1}) is the desired specific growth rate; and $Y_{X/C}$ is the yield (g g^{-1}) of biomass from the mixture of carbon substrates which was 0.66 g g^{-1}, experimentally determined from continuous fermentation by feeding nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25: 1: 0.05 at a specific growth rate of 0.25 h^{-1} (Chapter 3).

The mass of each carbon source required at time t was calculated according to the pre-defined ratio of the two substrates as follows:

$$S_{t-NA} = S_t \cdot f_{NA} \quad (2)$$

$$S_{t-G} = S_t \cdot f_G \quad (3)$$

The feeding ratio of nonanoic acid to glucose in this study was 1.25: 1 (w/w). Therefore, the mass fraction of nonanoic acid (f_{NA}) and that of glucose (f_G) in the total carbon source were 0.56 and 0.44, respectively.

Exponential substrate feeding began after a lag phase of approximately 5 h. Fermentations with a specific growth rate of 0.25 h^{-1} were conducted only under exponential feeding. Exponential feeding at 0.15 h^{-1} was conducted for 23.3 h before changing to a constant feed rate of 8 g L^{-1} h^{-1} to avoid nonanoic acid and acrylic acid overfeeding.

4.3.4 Analytical procedures

Duplicate samples of 10 mL culture broth were centrifuged at 6,000 $\times g$ for 15 min, washed and lyophilized and biomass concentration determined gravimetrically. Sample supernatants were analyzed for the concentrations of residual nutrients and acrylic acid. Glucose was measured colorimetrically after reacting with 4-hydroxybenzoic hydrazide under alkaline condition (Lever, 1972). Nonanoic acid was methylated in acidified methanol (Ramsay et al., 1991) and analyzed by a CP3900 Varian GC equipped with a

flame ionization detector. Phosphate was measured based on the reduction of phosphomolybdate to molybdene blue (Clesceri et al., 1999). Ammonium was determined by the phenol-hypochlorite method (Weatherburn, 1967). Acrylic acid was assayed by Hewlett-Packard GC equipped with a Cabowax®-PEG column after acidification with one tenth volume of 2 N hydrochloric acid (Qi et al., 1998).

PHA content and composition in the dry biomass samples were determined by methanolysis in 2 mL chloroform and 1 mL methanol which contained sulfuric acid (15%, v/v) as acidifying agent and benzoic acid (0.2%, w/v) as internal standard at 100^{o}C for 4 h. After which, 1 mL distilled water was vigorously mixed on a Fisher Vortex and left overnight for phase separation. One μL of the chloroform phase was injected into CP3900 Varian GC at a split ratio of 20. The injector and detector were maintained at 250 and 275^{o}C, respectively. The oven heating profile was: initial 90^{o}C for 0.5 min, 5^{o}C min^{-1} to 95^{o}C and hold for 0.5 min, 30^{o}C min^{-1} to 170^{o}C and hold for 2.5 min. The PHA standard was prepared by acetone extraction and methanol precipitation followed by three cycles of extraction and precipitation, as described by Jiang et al. (2006) and the monomeric composition characterized by GC and proton nuclear magnetic resonance at room temperature in a Bruker Avance 200 spectrometer using deuterated-chloroform containing 20 mg mL^{-1} PHA.

4.4 Results

4.4.1 Co-feeding nonanoic acid, glucose, and acrylic acid at a mass ratio of 1.25: 1: 0.01 and a μ of 0.25 h^{-1}

Feeding nonanoic acid (NA), glucose (G), and acrylic acid (AA) at a mass ratio of 1.25: 1: 0.01 and a specific growth rate of 0.25 h^{-1} produced 34 g L^{-1} dry biomass

containing a maximum of 56% PHA (Figure 4-1 (a)). Phosphate and ammonium were controlled at levels that had been previously shown to be sufficient but not inhibitory to cell growth (Sun et al., 2007). Before 13.6 h, nonanoic acid and glucose concentrations were very low, indicating that the carbon source was the only limiting factor (Figure 4-1 (b)). Uncontrollable foaming occurred at 15.8 h, accompanied by accumulation of acrylic and nonanoic acids in the reactor but glucose was entirely consumed. During the early stages of cultivation, the PHA contained 81 mol% HN which increased slightly to 84 mol% (Figure 4-1 (c)), much more than the 65 mol% HN produced without acrylic acid.

4.4.2 Co-feeding nonanoic acid, glucose, and acrylic acid at a mass ratio of 1.25: 1: 0.05 and a μ of 0.25 h^{-1}

In an attempt to further increase the HN content, the culture conditions were kept the same as above except that the amount of acrylic acid was increased five times. The results were similar to what is shown in Figure 4-1except that the residual nonanoic acid and acrylic acid accumulated earlier (starting at 12.4 h instead of 15.8 h). Again, there was uncontrollable foaming and only 17 g L^{-1} final biomass was produced (Figure 4-2 (a) (b)). However, the PHA content increased from 56 to 64% with 90 mol% HN in early samples with a slight increase to 92 mol% (Figure 4-2 (c)).

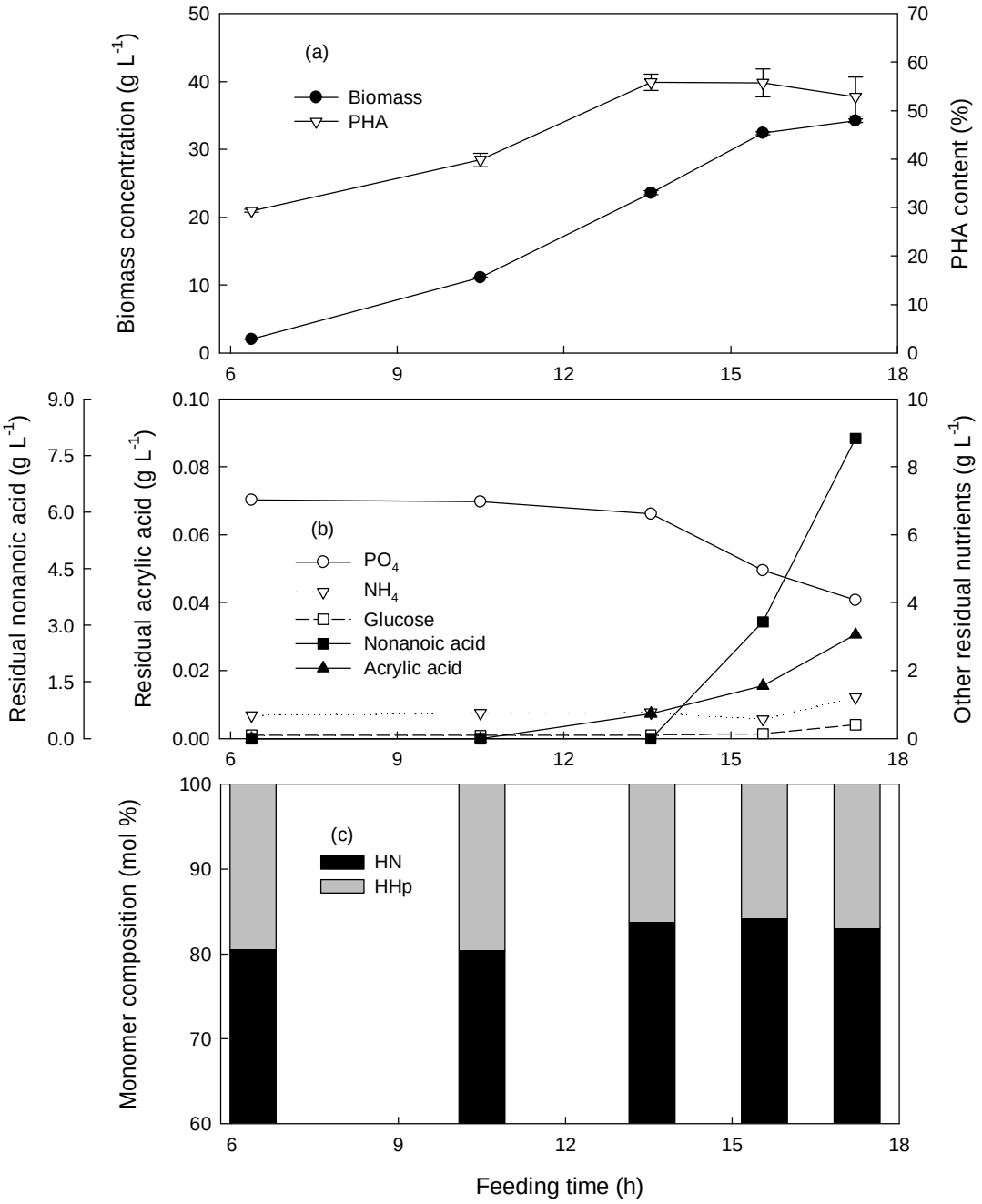

Figure 4-1 Cultivation of *P. putida* KT2440 by feeding nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25: 1: 0.01 and a specific growth rate of 0.25 h^{-1} in fed-batch fermentation. HN, 3-hydroxynonanoate; HHp, 3-hydroxyheptanoate. Symbols are at the average of two samples and error bars represent the range of the average.

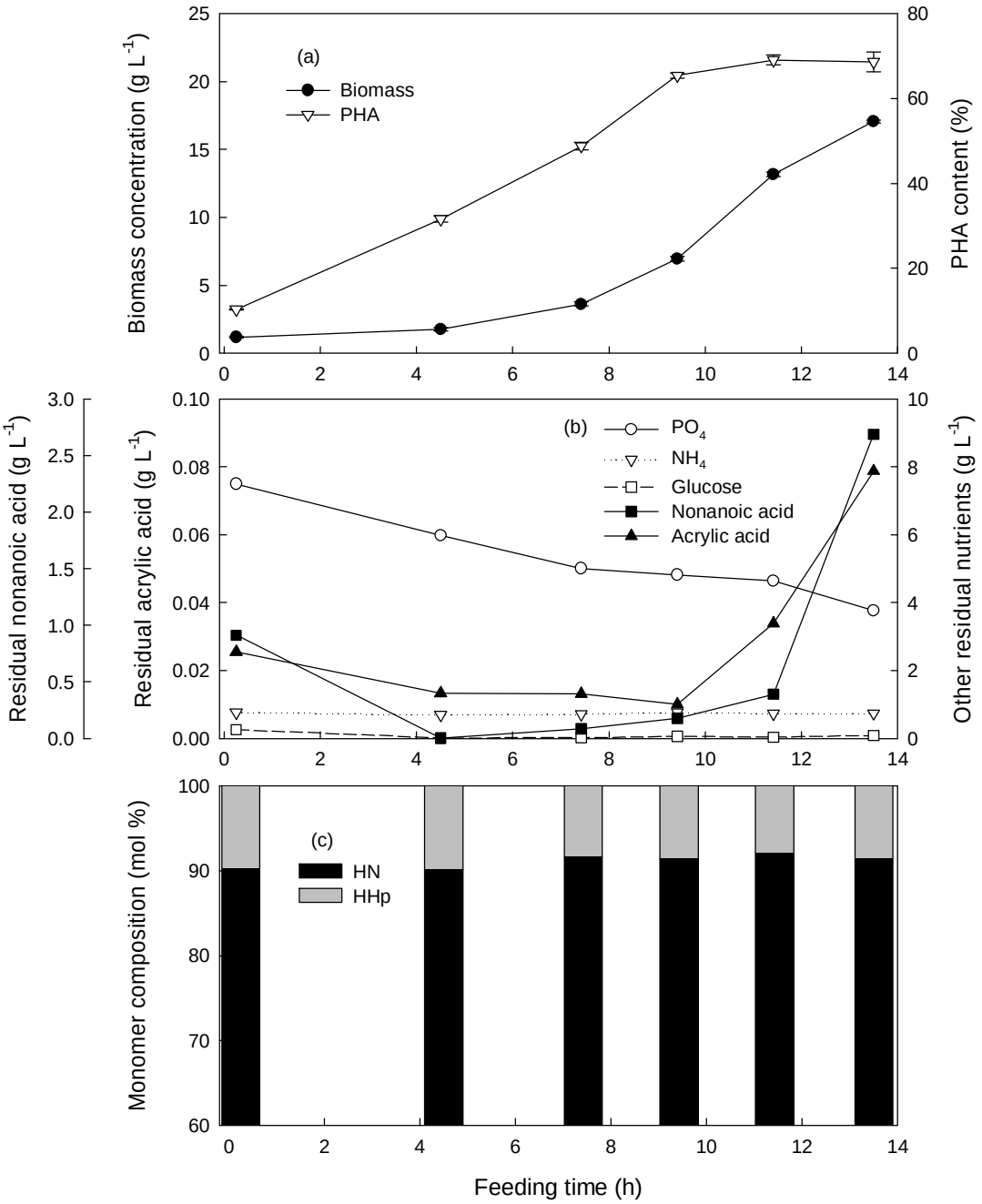

Figure 4-2 Cultivation of *P. putida* KT2440 by feeding nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25: 1: 0.05 and a specific growth rate of 0.25 h^{-1} in fed-batch fermentation. HN, 3-hydroxynonanoate; HHp, 3-hydroxyheptanoate. Symbols are at the average of two samples and error bars represent the range of the average.

4.4.3 Co-feeding nonanoic acid, glucose, and acrylic acid at a mass ratio of 1.25: 1: 0.05 and a μ of 0.15 h^{-1}

In order to avoid nonanoic acid and acrylic acid accumulation, the feeding program was adjusted to achieve a lower μ of 0.15 h^{-1} at a NA: G: AA feeding ratio of 1.25: 1: 0.05 then, at 23.3 h, a constant feed rate of 8 g L^{-1} h^{-1}. Under these conditions, a biomass concentration of 71.4 g L^{-1} was achieved (Figure 4-3 (a)). The PHA content increased in two steps, from 0 to 59.8% followed by a 10 h plateau, then following the change to the constant feed rate, there was a second increase from 58.1 to 75.5%.

From the beginning of the fermentation, there was constant foaming which became more severe at 12 h. At this time, the supplemental phosphate solution (20 mL) and trace element solution (30 mL) were sequentially added. Antifoam was added dropwise and the foam was gone after about a half hour. It can be seen from Figure 4-3 (b) that phosphate was maintained at non-limiting levels throughout the fermentation. Ammonium was automatically controlled to be in the range of 1~1.5 g L^{-1} as in the previous two fermentations. The residual concentration of glucose was slightly above zero. While there was a slight increase in the residual nonanoic acid concentration after 12 h, its concentration dropped after 16 h and was maintained below 0.5 g L^{-1} until near the end of the fermentation. Dissolved oxygen dropped to zero at 29.6 h despite a supply of 1 vvm pure oxygen and remained there. PHN containing about 88 mol% HN was obtained.

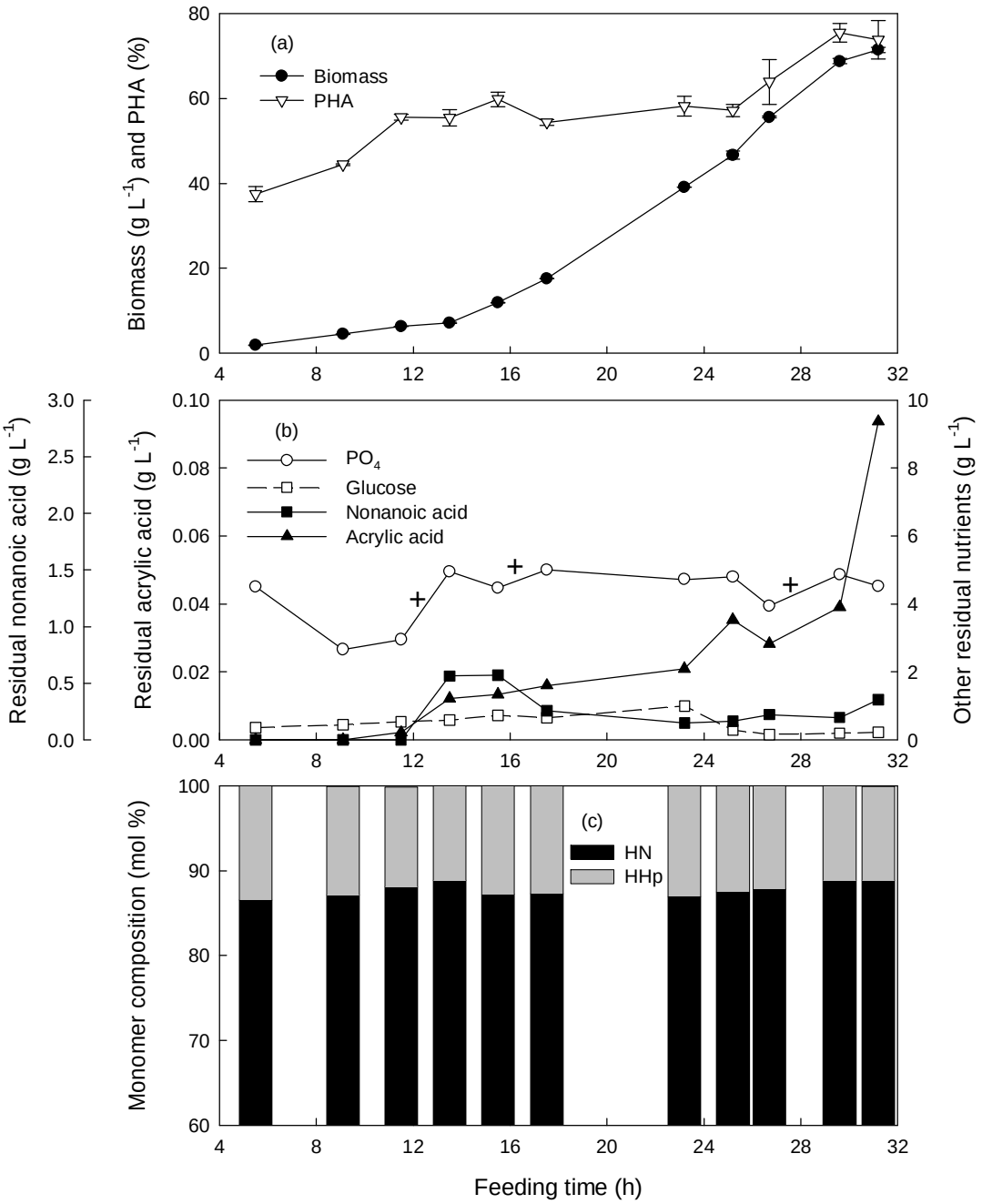

Figure 4-3 Cultivation of *P. putida* KT2440 by feeding nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25: 1: 0.05 and a specific growth rate of 0.15 h^{-1} in fed-batch fermentation. HN, 3-hydroxynonanoate; HHp, 3-hydroxyheptanoate. 20 mL supplemental phosphate solution was added at the times indicated by "+" above the phosphate curve. Symbols are at the average of two samples and error bars represent the range of the average.

4.4.4 Comparison of the three fed-batch fermentations

Despite the presence of different acrylic acid concentrations, growth at μ of 0.25 h^{-1} followed the same trend until foaming occurred (Figure 4-4). In contrast, PHA accumulation increased as the acrylic acid concentration increased. When μ was lower (0.15 h^{-1}), slower growth and polymer accumulation were observed compared to that at μ of 0.25 h^{-1} and NA: G: AA= 1.25: 1: 0.05.

The cumulative PHA productivity in all fermentations increased until the last or second to last sample point. The highest productivity of 1.8 g L^{-1} h^{-1} was obtained at a combination of the higher acrylic acid concentration and the lower growth rate (Figure 4-5).

Acrylic acid consumption was proportional to total biomass produced (Figure 4-6 (a)) with the highest slope observed at lower acrylic acid feed concentration. Although higher acrylic acid feed concentration was favorable for achieving higher HN content, a lower concentration was more efficient in terms of the enhanced HN production (Figure 4-6 (b)).

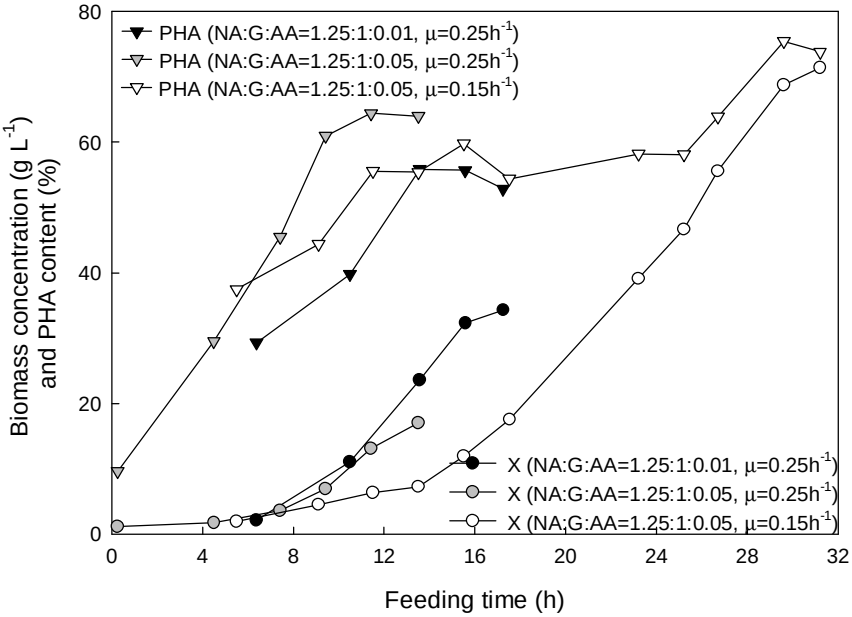

Figure 4-4 Effect of inhibitor feeding ratio and growth rate on cell growth and PHA synthesis in *P. putida* KT2440 by co-feeding nonanoic acid and glucose at the presence of acrylic acid. NA, nonanoic acid; G, glucose; AA, acrylic acid; X, biomass.

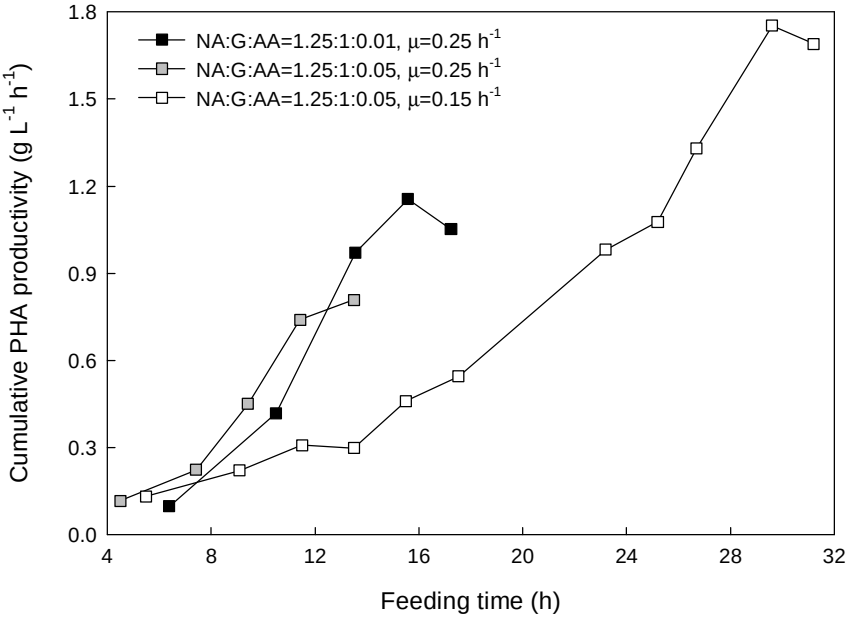

Figure 4-5 Cumulative PHA productivity in *P. putida* KT2440 under nonanoic acid and glucose co-feeding at the presence of acrylic acid in fed-batch fermentations.

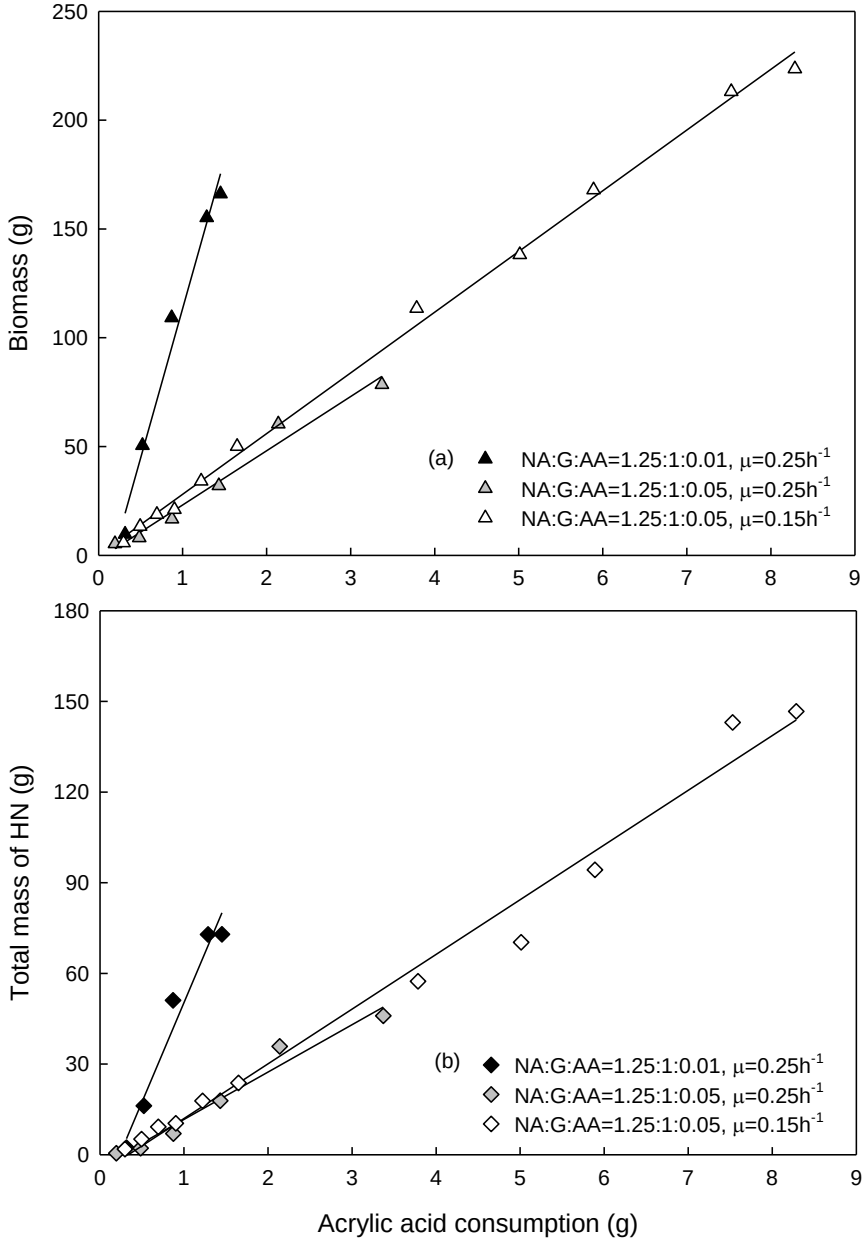

Figure 4-6 Biomass and total mass of HN as a function of acrylic acid consumed. HN, 3-hydroxynonanoate; NA, nonanoic acid; G, glucose; AA, acrylic acid.

4.5 Discussion

Control of the monomeric composition of MCL-PHA in fed-batch fermentation is novel and challenging. The application of acrylic acid, a β-oxidation inhibitor, is the key to flexible control of the monomeric composition of MCL-PHA. However, microbial cultivation using fatty acid substrates in the presence of acrylic acid has been previously demonstrated to result in poor cell growth and MCL-PHA accumulation both in our chemostat studies (chapter 3) and in the literature (Huijberts et al., 1994). The strategy of co-feeding a fatty acid (nonanoic acid in this study) and a carbohydrate (glucose in this study) is essential in obtaining a high cell density with high PHA content, especially in an efficient process. As the acrylic acid concentration increased in the co-substrate feed, at the same feed rate (μ = 0.25 h^{-1}), the accumulation of MCL-PHA increased as well as the proportion of HN monomers from 65 mol% (no acrylic acid) to greater than 92 mol% (at NA: G: AA = 1.25: 1: 0.05) (Table 4-1). The increase in the HN content probably reflects increasing β-oxidation inhibition with the increasing acrylic acid concentration.

Fed-batch fermentation was able to obtain the same inhibition as continuous cultivation since the monomeric composition and cumulative PHA productivity were similar (Table 4-1) at the same growth rate (μ = 0.25 h^{-1}) and NA: G: AA feeding ratio (1.25: 1: 0.05). However, in contrast to a continuous or batch cultivation where nutrients and other additives are either at steady state concentrations or consumed gradually, substrates in fed-batch fermentations may accumulate if they are consumed more slowly than the feed rate. Accumulation of toxic substances could be harmful since it may lead to cell death and uncontrollable foaming. About 3~4 g L^{-1} of nonanoic acid (Sun et al., 2006) and as little as 0.1 g L^{-1} acrylic acid (Appendix A) are toxic to *P. putida* KT2440.

In this study, the use of acrylic acid is even more challenging since nonanoic acid accumulation was accelerated by β-oxidation inhibition and occurred very quickly as seen in the two fermentations at the higher feed rate (μ = 0.25 h^{-1}). At a lower feed rate (μ = 0.15 h^{-1} followed by linear feeding), although there were two minor foaming events at 12 and 23 h accompanied by a noticeable but lower level of nonanoic and acrylic acid accumulation, foaming was controllable. This resulted in a longer fermentation with much higher biomass production (71.4 g L^{-1}), higher MCL-PHA accumulated (75.5%) with a reasonably high HN content (about 89 mol%) and the best MCL-PHA productivity (1.8 g L^{-1} h^{-1}) and yield of PHA from NA (0.78 g g^{-1}) (Table 4.1). This may be improved further by using a decaying substrate feeding strategy (Maclean et al., 2008).

Since acrylic acid consumption was linearly related to cell growth (Figure 4-6 (a)) and residual acrylic acid concentration in the reactor appears to follow that of nonanoic acid (Figures 4.1 to 4.3), it is likely that acrylic acid was continuously taken up by the cells. Therefore, the feeding of acrylic acid should be proportional to cell growth so as to impose a certain level of inhibition if a constant monomeric composition is desired. The combination of an appropriate concentration of the β-oxidation inhibitor and a growth rate which avoids toxic accumulation of both nonanoic and acrylic acid enhanced growth and PHA accumulation as well as controlled the monomeric composition.

This is the first time that this combination has been demonstrated in high-cell-density production of MCL-PHA in a fed batch process.

Table 4-1 Comparison of fermentations producing PHN using *P. putida* KT2440

Specific growth rate (h^{-1})	NA:G:AA* feeding ratio (w/w/w)	Fermentation type	Biomass ($g\ L^{-1}$)	PHA (%)	HN (mol%)	HHp (mol%)	Yx/c** ($g\ g^{-1}$)	$Y_{PHA/NA}$** ($g\ g^{-1}$)	PHA productivity*** ($g\ L^{-1}\ h^{-1}$)	Reference
0.25	1:1:0	Fed-batch	71.0	56.0	65.0	35.0	0.62	0.66	1.4	Sun et al., 2009
0.25	1.25:1:0.05	Chemostat	5.8	51.7	91.3	8.7	0.66	0.61	0.7	Chapter 3
0.25	1.25:1:0.01	Fed-batch	34.3	55.7	84.3	15.7	0.62	0.68	1.2	This study
0.25	1.25:1:0.05	Fed-batch	17.1	64.4	92.2	7.8	0.53	0.68	0.8	This study
0.15	1.25:1:0.05	Fed-batch	71.4	75.5	88.9	11.1	0.62	0.78	1.8	This study

* NA, nonanoic acid; G, glucose; AA, acrylic acid; HN, 3-hydroxynonanoate; HHp, 3-hydroxyheptanoate.
** $Y_{X/C}$, yield of biomass from total carbon substrate; $Y_{PHA/NA}$, yield of PHA from nonanoic acid.
*** PHA productivity was calculated as the cumulative PHA productivity based on feeding time.
**** Biomass, PHA content, HN and HHp percentage, and PHA productivity were reported as the highest values during the fermentations, while $Y_{X/C}$ and $Y_{PHA/NA}$ were reported as the slopes of the trend line that was drawn from all points of each fermentation.

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Chapter 5

Solvent extraction of MCL-PHA from *Pseudomonas putida* KT2440

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5.1 Abstract

A methodology was developed for the extraction of medium-chain-length poly-3-hydroxyalkanoates (MCL-PHA) from *Pseudomonas putida*. It was determined that if dry *P. putida* biomass containing MCL-PHA was washed in 20 volumes of methanol for 5 min followed by Soxhlet extraction in 10 volumes of acetone for 5 h that almost all of the PHA could be recovered with no detectable loss of molecular weight. Biomass containing higher amounts of PHA required less methanol during the pretreatment step but more acetone in the solvent extraction step than biomass containing less PHA. Further purification was achieved by redissolving the PHA in acetone and reprecipitating in cold methanol. UV spectroscopy at 241 and 275 nm was used as an indication of product purity.

5.2 Introduction

Medium-chain-length polyhydroxyalkanoates (MCL-PHAs) are polyesters produced by rRNA group I *Pseudomonas* species (Lageveen et al., 1988). The MCL-PHAs contain 6 to 14 carbons in their subunits and possess attractive properties such as low melting points, high elasticity, and biodegradability (Gagnon et al., 1992; Schirmer et al., 1993). These properties have led to suggestions for their application in paint formulations, other coatings, as controlled release compounds and in tissue engineering (Kurth et al., 2002; Sodian et al., 2000; Van der Walle et al., 1999). They can be depolymerized to chiral

monomers for potential use in the synthesis of antibiotics (Schnurrenberger et al., 1987), vitamins (Chiba and Nakai, 1985), and other bioactive compounds (Seebach and Zuger, 1982).

MCL-PHA only emerged as a distinct group of PHA polyesters two decades ago (Desmet et al., 1983). The short-chain-length PHA (SCL-PHA) contain less than 6 carbons in their subunits and some SCL-PHA copolymers have been commercially available since the 1980s (Holmes, 1985). SCL-PHA have been much more extensively studied. They are typically rigid, highly crystalline (Marchessault and Yu, 2001), and are quite different from MCL-PHAs which are regarded as thermoplastic elastomers. A major factor hindering the study of MCL-PHA is lack of availability. MCL-PHA fermentation technology has greatly advanced in recent years (Diniz et al., 2004; Jung et al., 2001). However, relatively little work has been reported on methodologies for separating these intracellular materials from the other biomass components.

As with SCL-PHA, most MCL-PHA separation methods employ solvent extraction or non-PHA biomass digestion. Digestion of biomass other than PHA (DeKoning and Witholt, 1997; DeKoning et al., 1997) typically consists of heat treatment, enzymatic solubilization, and surfactant washing. Drawbacks include expense, complexity and binding of materials such as surfactants to the PHA granules. Several enzymatic, centrifugation and washing steps are typically needed to achieve acceptable purity.

Solvent extraction is simpler in terms of the number of steps employed but there are still important choices to be made when designing a process. A PHA extraction process invariably involves three steps. These are biomass pretreatment, solvent extraction, and polymer purification (Figure 5-1). The pretreatment step may incorporate enzymes to

degrade proteins and DNA, heating to denature these macromolecules, surfactants to remove lipids and/or solvents to remove water and polar lipids. Pretreatment is critical to the subsequent solvent extraction process as it affects the accessibility and the solubility of the PHA. Solvent extraction of SCL-PHA most often involves chlorinated hydrocarbons such as chloroform (Ramsay et al., 1994), but MCL-PHA is soluble in a much broader solvent range, so cheaper and less toxic solvents may be used. Thus, although there have been many publications dealing with solvent extraction of SCL-PHA, these are only indirectly applicable to MCL-PHA extraction. Acetone is the most commonly used solvent in MCL-PHA extraction while methanol is usually employed as the MCL-PHA non-solvent both for biomass pretreatment and PHA precipitation from solution in acetone. The objective of the present study was to evaluate the impact of biomass pretreatment and subsequent acetone extraction on the recovery and purity of MCL-PHAs. Since NaOH is frequently used in biomass pretreatment, its effect on subsequent extraction efficiency was examined and compared to methanol. Since *Pseudomonas putida* has become the most frequently cited species for MCL-PHA production, *P. putida* KT2440 biomass was used throughout. This is the first detailed report of a solvent extraction methodology for MCL-PHA.

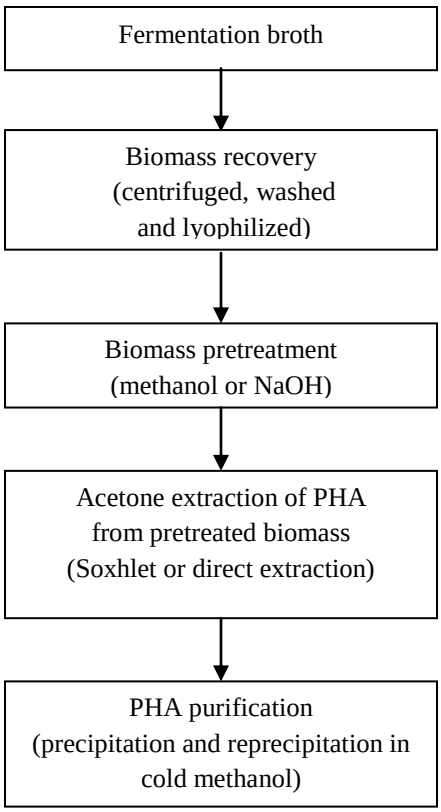

Figure 5-1 Schematic flowchart of the MCL-PHA recovery process in this study.

5.3 Materials and methods

5.3.1 MCL-PHA biomass and reagents

Pseudomonas putida KT2440 (ATCC 47054) was grown in fed-batch culture as described by Sun et al. (2006) with glucose or nonanoic acid as the sole carbon source during the growth phase and either glucose or nonanoic acid or a mixture of glucose and nonanoic acid during the accumulation phase. The biomass was recovered by centrifugation in a Sorval centrifuge at 10,400 ×*g*, washed twice with distilled water, lyophilized and stored at -20^{o}C until used. Five batches with different PHA content and monomeric compositions (Table 5-1) were produced.

Methanol (Reagent ACS), acetone (Certified ACS) and sodium hydroxide (Certified ACS) used throughout the experiments were purchased from Fisher Scientific. Other reagents used in gas chromatography analysis included chloroform (ACS HPLC grade) from Aldrich, benzoic acid (Reagent ACS) from Anachemia Ltd., and sulfuric acid (Reagent ACS) from Fisher Scientific.

Table 5-1 PHA content and monomeric composition of different batches of biomass used in this study

% PHA in biomass	Carbon source during growth/accumulation	PHA monomers	Experiment
4	Glucose/Glucose	HO, HD	Methanol to biomass ratio
20	Glucose/Glucose and nonanoic acid	HHp, HO, HN, HD	NaOH versus methanol pretreatment
10	Glucose/Glucose and nonanoic acid	HHp, HO, HN, HD	Acetone to biomass ratio
66	Nonanoic acid/ Nonanoic acid	HHp, HN	Acetone to biomass ratio
27	Nonanoic acid/ Nonanoic acid	HHp, HN	Soxhlet versus direct extraction

HHp, 3-hydroxyheptanoate; HN, 3-hydroxynonanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate;

5.3.2 Biomass pretreatment

5.3.2.1 Comparison of NaOH and methanol pretreatment

Triplicate 1 g samples of biomass were mixed with either 20 ml of methanol, or 0.1 N NaOH or water (as a control) at 22 $\pm 1^{o}$C for 5 min, 30 min, 2 h, or 5 h. Biomass was

recovered by centrifugation in a Beckman centrifuge at 10,000 $\times g$ for 30 min, washed in distilled water and recentrifuged at 15,000 $\times g$ for 15 min. PHA content was determined for all supernatant and biomass samples and PHA M_W determined for the latter. After pretreatment, the biomass samples were lyophilized and PHA extracted at 160 rpm for 22 h into 10 ml acetone then filtered through Fisherbrand® G6 glass-fiber filters. The PHA acetone extract was air-dried before GC analysis.

5.3.2.2 Effect of methanol to biomass ratio

To evaluate the efficiency of methanol to extract impurities (cell components other than PHA), a biomass with low PHA content (4.8%) was used. Different ratios of methanol (ml) to biomass (g) (5, 8, 10, 20, 50, 100) were mixed at 22 $\pm 1^{o}$C and 160 rpm for 12 h. After filtration, the biomass was air-dried to a constant mass. The mass of cellular material dissolved by methanol was calculated as the difference in biomass dry weight before and after methanol treatment.

5.3.3 Acetone extraction of PHA

5.3.3.1 Effect of acetone to biomass ratio

After methanol pretreatment (20 ml methanol to 1 g biomass for 30 min at 22 $\pm 1^{o}$C and 160 rpm), different amounts of acetone were added to 2 g biomass (weight before methanol wash) containing either 10 or 66% PHA to have volume to weight ratios of 2.5, 5, 10, 15, 20 ml g^{-1}. After mixing for 24 h at 170 rpm and 22 $\pm 1^{o}$C, the PHA solubilized in acetone was separated by filtration and the residual biomass washed with 20 ml fresh acetone. Both volumes of acetone were combined and rotary evaporated at 50 $\pm 1^{o}$C to about 2 ml and the evaporator rinsed with 1 ml fresh acetone. The pooled samples were added dropwise to 10 volumes (20 ml) cold methanol with vigorous stirring. The

precipitate was recovered by filtration, air-dried for 4 days, then weighed and analyzed by GC.

5.3.3.2 Comparison of Soxhlet and direct extraction methodologies

For Soxhlet extraction and direct extraction under ambient conditions, 2.5 and 5 g biomass respectively containing 27% PHA (dry weight) were pretreated with methanol (20 ml methanol per g biomass for 30 min at 22 $\pm 1^{o}$C and 160 rpm) in duplicate experiments. The 5 g sample was then extracted with 10 volumes (50ml) of acetone at 170 rpm and 22 $\pm 1^{o}$C for 5 min, 1 h, 6 h, and 24 h.

For Soxhlet extraction, the 2.5 g methanol pretreated biomass was extracted into 50 ml acetone at its boiling point (56^{o}C) for 15min, 30min, 1h or 5 h. For both methods, the PHA solubilized in acetone was recovered, concentrated by rotary evaporation for the same length of time and precipitated as described above.

5.3.4 PHA purification

PHA (0.2 g) recovered under ambient extraction (as described in section 5.3.2) was dissolved in 1 ml acetone and reprecipitated in 50 ml ice-cold methanol. The dissolution-precipitation cycle was repeated three times. After each cycle, a 0.02 g dry PHA sample was dissolved in 4 ml chloroform and a spectral scan was performed using a UV-Visible spectrophotometer (Spectronic Unicam UV1) with chloroform as the blank. Solid samples were analyzed by infrared spectroscopy (Avatar 320 FT-IR system) with a Nicolet ATR. All analyses were performed with at least duplicate samples obtained from independent experiments.

5.3.5 PHA recovery and purity

PHA samples were prepared as described by Braunegg et al. (1978) using 15% (v/v) concentrated sulfuric acid instead of 3% and quantified with a HP 5890 GC using the conditions of Ramsay et al. (1991). Purity was determined from a known mass of sample from the PHA analysis using benzoic acid as the internal standard. From a known amount of PHA in the biomass, the percent PHA recovered was calculated based on the purity of the total mass recovered. Each duplicate sample from the above experiments was analyzed by GC at least twice.

The weight average molecular weight of the polymer prepared in distilled tetra-hydrofuran (THF) was analyzed at 40^{o}C using a Waters 2695 Gel Permeation Chromatograph equipped with four Styragel columns of pore sizes 100, 500, 10^3, and 10^4 Å coupled to a Water 410 differential refractive index detector, THF as the mobile phase at 1 ml min^{-1} and polystyrene standards.

5.4 Results

The recovery process for MCL-PHA via solvent extraction is shown in Figure 5-1. This study focused on two major steps: biomass pretreatment and PHA extraction into acetone. A purification step was also examined.

5.4.1 Biomass pretreatment

5.4.1.1 Comparison of NaOH and methanol pretreatment

Pretreatment of biomass is intended to make the biomass more amenable to solvent extraction and also to remove contaminants before they can be extracted into the solvent with the PHA. Weighing the dry biomass before and after pretreatment showed that exposure to base removed the largest amount of cellular material and produced a high

PHA content in the resulting biomass (Figure 5-2). However a substantial amount of PHA was lost during NaOH pretreatment probably related to the increase in viscosity (due to cell lysis) not observed with methanol or the control (water). The increased viscosity made centrifugation more difficult. Methanol pretreatment achieved its maximum polymer purity (24%) and removal of cell components other than PHA within 5 min of contact time (Figure 5-2). In contrast, about 2 h of contact with NaOH was required to achieve a maximum in biomass removal and PHA purity (44%).

Since the purpose of pretreatment is to improve the PHA purity and recovery in subsequent solvent extraction, additional samples (i.e. ones not destroyed by molecular weight analysis) that had been washed in methanol, NaOH and water, were extracted with acetone. Although methanol or NaOH pretreatments decreased the amount of PHA recovered after acetone extraction compared to the control which was washed with water (Figure 5-3), PHA purity greatly increased.

GPC analysis showed the M_w of the bulk of MCL-PHA in all samples was 82 ± 1 kDa (Figure 5-4) with a polydispersity index of 1.82 (1.86 for methanol pretreatment). There was no significant M_w decrease with any pretreatment. However, minor peaks with a retention time of 30-38 min, representing low-molecular-weight MCL-PHA, were eliminated with both methanol and NaOH pretreatments.

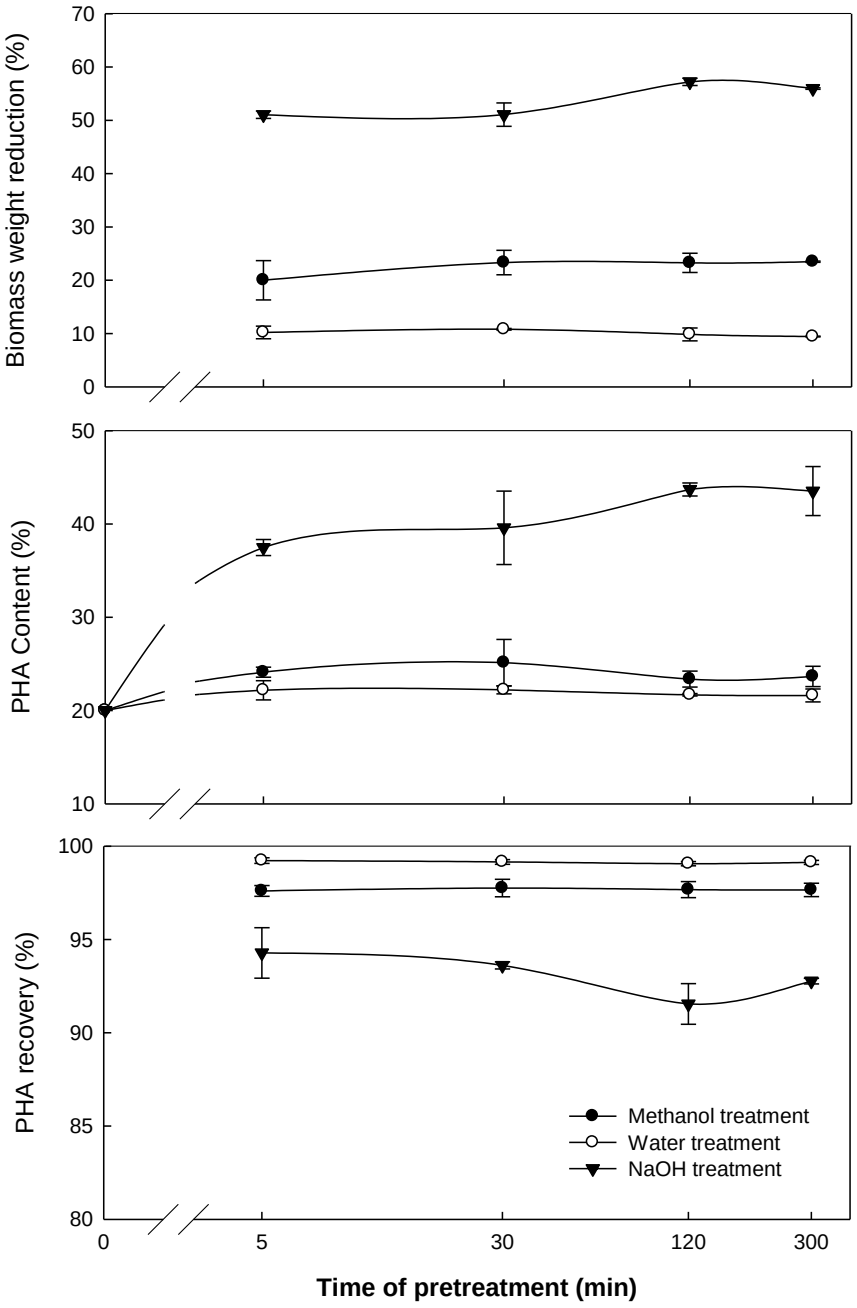

Figure 5-2 Effect of NaOH or methanol pretreatment on *P. putida* biomass containing MCL-PHA. Symbols and error bars represent mean and range respectively of duplicate samples. Lines are to guide the eye.

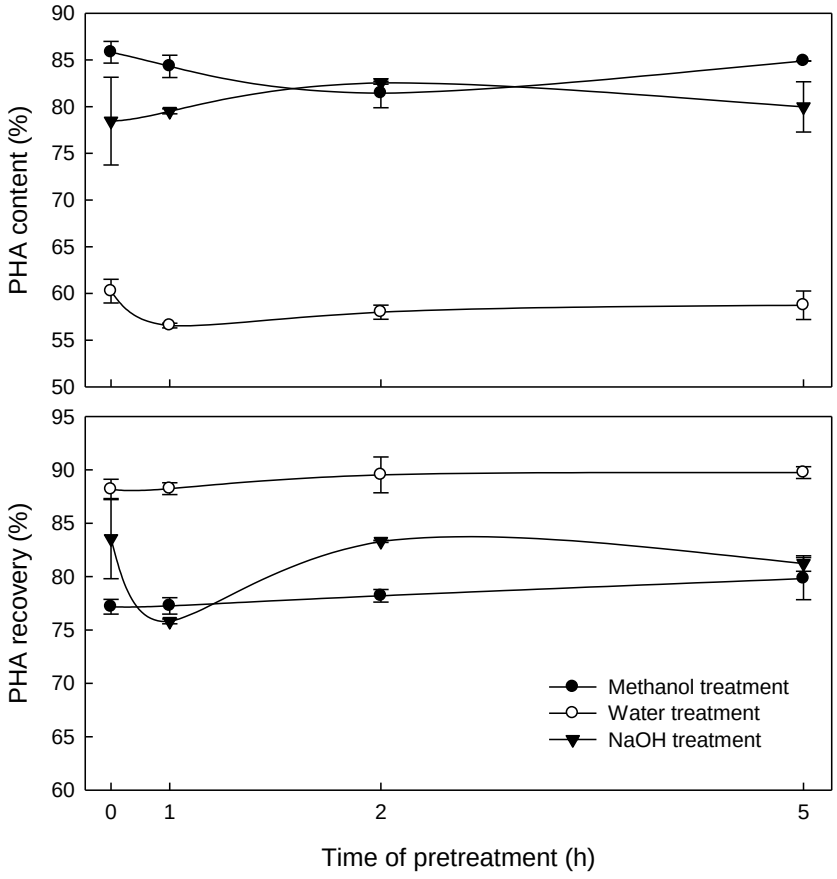

Figure 5-3 Effect of extraction of MCL-PHA into acetone of the NaOH or methanol pretreated biomass of Figure 5-2 on PHA recovery and purity. Symbols and error bars represent mean and range respectively of duplicate samples. Lines are to guide the eye.

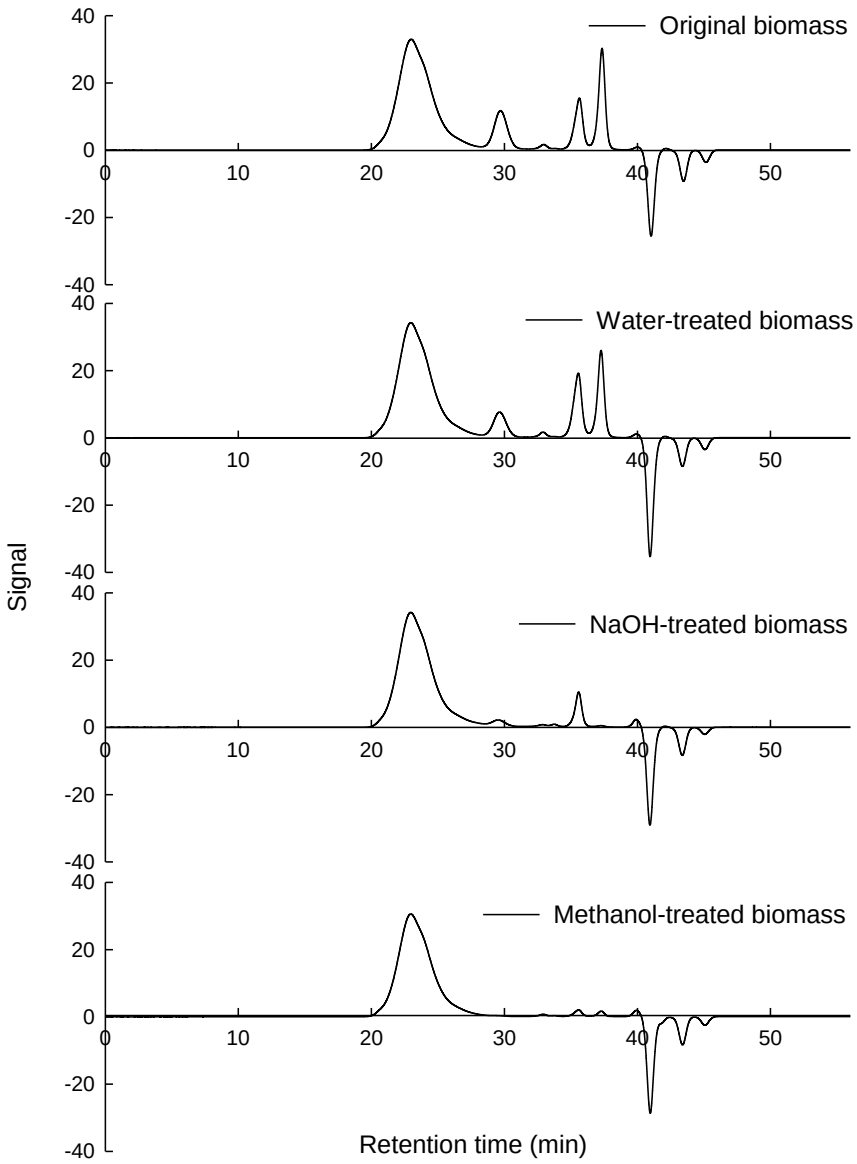

Figure 5-4 Gel permeation chromatographs of the MCL-PHA recovered from the experiment presented in Figure 5-2.

5.4.1.2 Methanol to biomass ratio

Increasing the methanol to biomass ratio extracted more impurities (Figure 5-5). The effectiveness of the amount of material extracted per volume of methanol used decreased greatly around a methanol to biomass ratio of 18 ml g^{-1}. This ratio (shown as the intersection of the two tangent slopes in Figure 5-5) gave a good compromise between maximum removal and the amount of solvent required to achieve removal. Since the original biomass contained only 4.8% PHA, it can be concluded that a methanol to residual biomass (total biomass minus PHA) ratio of about 20 is suitable for pretreatment of *P. putida* biomass. This biomass was grown on glucose. Growth on other substrates such as nonanoic or oleic acids may significantly affect the lipid quantity and composition, and affect the amount of methanol required for optimal pretreatment.

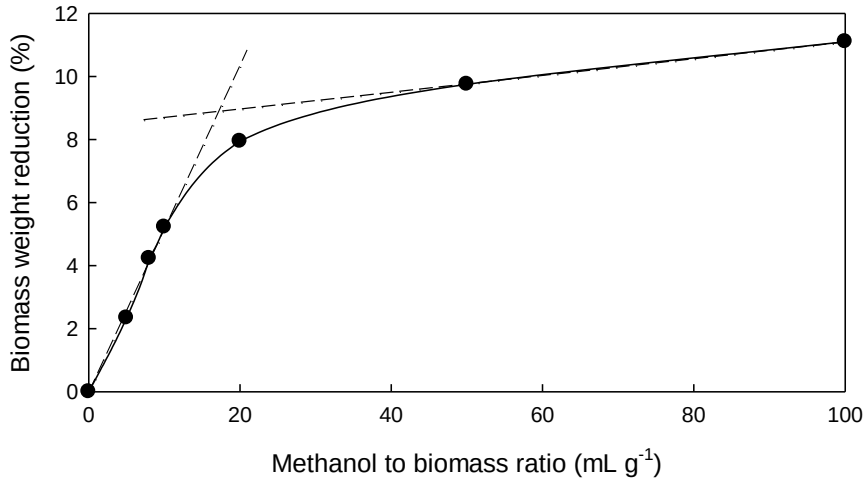

Figure 5-5 Effect of methanol to biomass ratio on removal of cellular materials other than PHA in the methanol pretreatment step. The intersection of the tangents was used to estimate an optimal ratio (about 18 mL g^{-1}). Lines are to guide the eye.

5.4.2 Acetone extraction after methanol pretreatment

5.4.2.1 Acetone to biomass ratio

Methanol pretreated MCL-PHA biomass was extracted by mixing with acetone under ambient conditions (direct extraction). Increasing the ratio of acetone to biomass increased the amount of PHA recovered in the biomass containing 66% PHA but had no effect on the biomass containing only 10% PHA (Figure 5-6). This indicates that the quantity of PHA to be extracted (rather than the amount of biomass) determines the quantity of solvent required. PHA recovery from the biomass containing 66% PHA attained a maximum of 82% at an acetone to biomass ratio of about 10 ml per g of dry biomass. This corresponds to an acetone to PHA ratio of 15.2 mL g^{-1}. Methanol pretreatment should also affect the amount of acetone required since it eliminates contaminants that could be co-extracted with the PHA.

The PHA purity decreased as the amount of acetone used increased especially with the low PHA-content biomass (Figure 5-6). These results indicate that there is a maximum in the extraction process where the greatest amount of PHA can be extracted with the least amounts of contaminants.

5.4.2.2 Direct, ambient extraction compared with Soxhlet extraction in acetone

Soxhlet extraction at 56^{o}C (boiling point of acetone) was much more effective at extracting MCL-PHA from methanol pretreated biomass than direct extraction under ambient conditions (Figure 5-7). Since the concentration gradient is always high during Soxhlet extraction and since the extraction temperature was slightly higher, virtually all the MCL-PHA (~99%) was extracted from the pretreated biomass after 5 h of treatment compared to 62% recovery after 24 h under ambient conditions. Unexpectedly the final PHA purity was similar using both techniques. Unlike direct extraction under ambient

conditions, PHA purity increased throughout the Soxhlet extraction process. There was no significant change in M_w during either process.

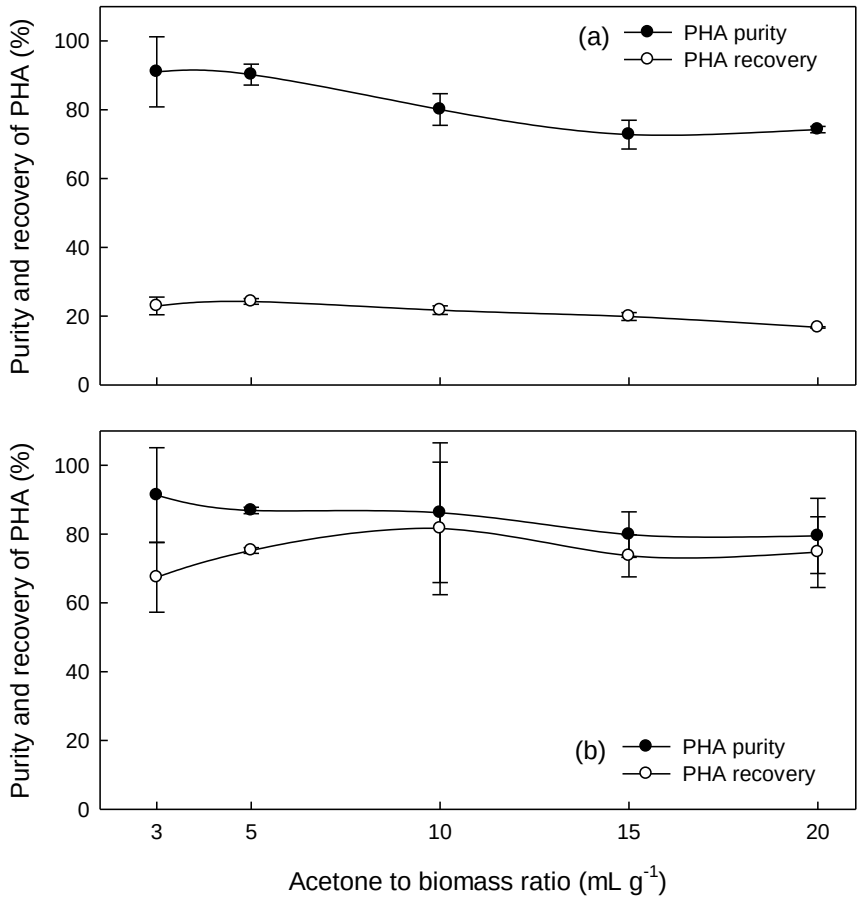

Figure 5-6 Effect of acetone to biomass ratio on purity and recovery of PHA of methanol pretreated biomass containing 10% PHA (a) and 66% PHA (b). Symbols and error bars represent mean and range respectively of two separate GC analyses of one sample. Lines are to guide the eye.

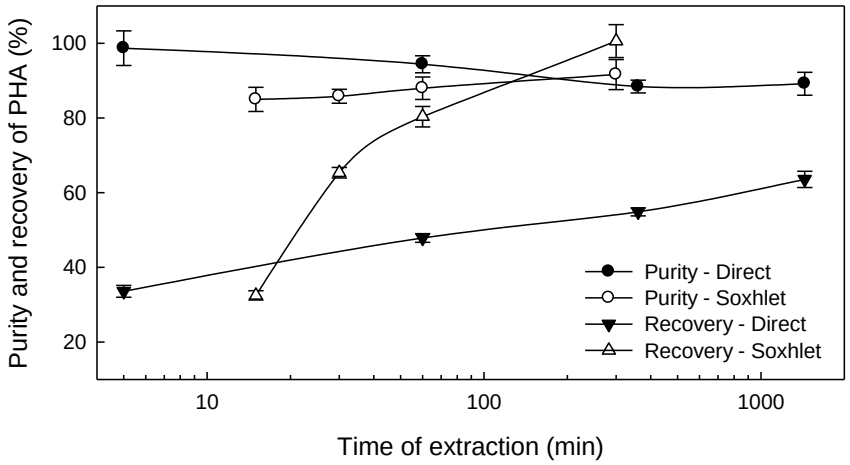

Figure 5-7 Comparison of Soxhlet extraction at 56 $\pm 1^{o}$C with direct extraction at 22 $\pm 1^{o}$C in acetone of methanol pretreated biomass on recovery and purity of PHA. Symbols and error bars represent mean and range respectively of duplicate samples. Lines are to guide the eye.

5.4.3 Impurities in PHA recovered

PHA samples were recovered after direct acetone extraction and after one to four cycles of dissolution in acetone followed by precipitation in cold methanol. The IR spectra of these samples showed the characteristic carbonyl peak of PHA at 1726 cm^{-1} with no differences between the purified and unpurified samples. UV spectroscopy of these samples dissolved in chloroform produced peaks at 241 and 275 nm (Figure 5-8). As the purity of the MCL-PHA increased with each successive dissolution-precipitation, the size of the peaks at 241 and 275 nm diminished. Nucleic acids and aromatic amino acids are known to absorb around 275 nm but further identification of the contaminants was not pursued.

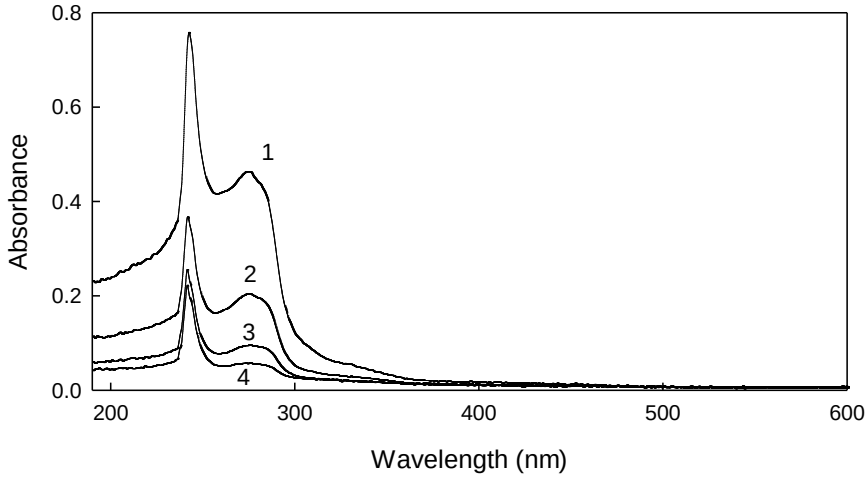

Figure 5-8 Comparison of the spectral scans of purified and unpurified MCL-PHA in chloroform. Samples were recovered by methanol pretreatment followed by direct extraction into acetone at 22 ± 1°C and precipitation in cold methanol (1). Lines (2), (3), and (4) represent the scans after each subsequent dissolution-precipitation cycle intended to purify the polymer.

5.4.4 PHA lost during recovery

A PHA balance of a recovery process in which methanol pretreatment preceeded direct acetone extraction under ambient conditions then precipitation in cold methanol was conducted to determine where PHA was lost during the process. For the biomass containing 66% PHA, the total amount of PHA detected from all steps of the separation process was 94% of the initial PHA (Table 5-2) but for the 10% PHA biomass the total amount detected was only ~74%. This is likely due to the inaccuracy involved in working with a much smaller quantity of PHA. Interestingly, almost half of the original PHA remained unextracted in the residual biomass when the original PHA content was 10% but only about 5% was unextracted when the original PHA content was higher (66%, Table 5-2). Even though almost 100% of the original PHA could be recovered from the 66% PHA biomass by Soxhlet extraction, it appears that a certain amount of PHA may be tightly bound to the biomass causing difficulty in direct extraction. This has been

previously observed with the SCL-PHA of *Ralstonia eutropha* where a small percentage of the PHA was never extracted despite repeated extractions in chloroform (per. comm. Bruce Ramsay).

Table 5-2 PHA balance of a recovery process in which the biomass was pretreated with methanol followed by direct acetone extraction under ambient conditions before precipitation in cold methanol

Initial % PHA in biomass	% of initial PHA in				% recovered of initial PHA
	solution after methanol pretreatment	solution after methanol precipitation	Residual biomass	PHA recovered	
10	3.3 ± 0.4	3.8 ± 2.7	48.8 ± 1.9	18.0 ± 3.7	73.9 ± 0.6
66	0.8 ± 0.1	1.2 ± 0.2	5.3 ± 0.4	86.6 ± 5.4	94.0 ± 5.4

The residual biomass was recovered by filtration. Error is reported as standard deviation of triplicate samples.

5.5 Discussion

The objective of this study was to evaluate the conditions of biomass pretreatment as well as the subsequent acetone extraction on the recovery and purity of MCL-PHA from *P. putida* biomass. Although NaOH was successfully used in SCL-PHA recovery from another Gram negative bacterium, *Escherichia coli* (Choi and Lee, 1999), this pretreatment induced cell lysis in *P. putida* leading to an increase in viscosity and loss of PHA. Heat treatment to coagulate the macromolecules responsible for the increased viscosity may aid recovery but a significant loss of polymer can still be expected. Methanol washing, as demonstrated in Figure 5-2 and Figure 5-3 and Table 5-2, results in very little loss of PHA from the biomass, yet it gives a similar final recovery as NaOH pretreatment if direct mixing with acetone is used in the extraction process. If Soxhlet

extraction is used, almost all of the PHA from the methanol pretreated biomass can be recovered. Unlike NaOH, methanol pretreatment effectively dries the biomass before extraction. This drying is thought to enhance subsequent PHA recovery using solvents. Both NaOH and methanol pretreatment eliminated low-molecular-weight PHA molecules (probably oligomers), but did not significantly decrease the M_w of the bulk polymer. The low M_W PHA may be soluble in methanol and may have been hydrolyzed by NaOH into water-soluble monomers. Overall, methanol is more suitable for pretreatment of *P. putida* biomass, and use of water washing as a control showed that pretreatment was highly beneficial.

Soxhlet extraction was far more effective than direct contact with acetone, although countercurrent extraction would likely be used if this material were to be produced at an industrial scale. Although Soxhlet extraction of SCL-PHA using chlorinated solvents has been shown to cause significant polymer degradation (Ramsay et al., 1994), there was no detectable loss of M_w during Soxhlet extraction of MCL-PHA in acetone in this study. This cannot be attributed to the difference in polymer structures which are very similar except for the length of the side chains. Thermal degradation of MCL-PHA in chloroform at 73 ± 2^oC proved to be faster than that in acetone or tetrahydrofuran (Appendix C). It was possible that the chlorinated solvents such as chloroform catalyze the depolymerization of MCL-PHA.

Detection of key contaminants is typically required if materials are to be used for medical applications. UV spectroscopy may be of some use in monitoring purity as further purification (i.e. reprecipitation in ice-cold methanol) diminished peaks at 241 and

275 nm[1]. Interestingly the carbonyl peak of the MCL-PHA absorbed at 1726 cm^{-1}, while Hong et al. (1999) reported that SCL-PHA absorbs at this wavelength and MCL-PHA should absorb at 1744 cm^{-1}.

The results of this study lead to recommendations for the isolation of high M_W MCL-PHA from *P. putida* biomass. Pretreatment of 1 g of residual *P. putida* biomass (dry biomass weight minus PHA) with 20 ml of methanol for 5 min should be followed by Soxhlet extraction in 10 ml of acetone for 5 h. Subsequently the solution should be concentrated by rotary evaporation before precipitation into cold methanol while mixing. Repeated dissolution in acetone followed by precipitation in methanol will aid in purification which may be monitored by UV spectroscopy at 241 and 275 nm. Further optimization of this recovery process should include a study of solvents other than acetone and of non-solvents other than methanol.

[1] Other quantitive analyses for possible contaminants were performed as described in Appendix D but not included in this chapter.

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Chapter 6

Recovery of medium-chain-length poly(3-hydroxyalkanoates) from *Pseudomonas putida* KT2440 by NaOH digestion

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6.1 Abstract

A method for the recovery of medium-chain-length polyhydroxyalkanoate (PHA) from *Pseudomonas putida* KT2440 was developed using NaOH digestion. PHA purity was affected by several factors including NaOH concentration, temperature, digestion time and the number of treatment cycles. PHA purity of about 85% was attained after 70 g/L biomass containing 66% PHA was treated with 0.2 N NaOH at 22 $\pm$ 1^{o}C for 2 h or with 0.1 N at 80^{o}C for 15 min. However, a sequential treatment of 0.2 N NaOH at 22 $\pm$ 1^{o}C for 2 h followed by 0.1 N NaOH at 80 $\pm$ 1^{o}C for 15 min improved the PHA purity to 94.7% with a recovery efficiency of 88%. Under these conditions, the digestion had a negligible effect on PHA molecular weight.

6.2 Introduction

Since the discovery of poly(3-hydroxybutyric acid) (PHB) in 1926, the poly(3-hydroxyalkanoate) (PHA) family has expanded to more than a hundred different monomers (Steinbuchel and Lutke-Eversloh, 2003). They are categorized into three groups based on the chain length of the monomers. Short-chain-length (SCL) PHAs contain 4 to 5 carbons in their repeating units and are typically crystalline thermoplastics. Medium-chain-length (MCL) PHAs have 6 to 14 carbons in their repeating units and resemble elastomers. SCL-MCL-PHA generally consists of 3-hydroxybutyrate and other MCL monomer constituents. Naturally occurring PHAs are biodegradable (Jendrossek et

al., 1996), non-cytotoxic, and resistant to gas and moisture permeation. They can be used to make packaging, coatings (Van der Walle et al., 1999), hygiene products (Holmes, 1985), drug carriers (Pouton and Akhtar, 1996) and tissue engineering materials (Sodian et al., 2000). Other potential applications can be developed since the polymer properties can be modified by changing the monomeric composition (Steinbuchel and Valentin, 1995), by functional group grafting (Kurth et al., 2002; Lee et al., 2000), and by post-synthetic treatment (Zhang et al., 2000).

MCL-PHA has been studied less than SCL-PHA, and although, it has many desirable properties, its production and downstream recovery are more challenging. Most separation methods have been developed for SCL-PHA. Property differences between MCL- and SCL-PHA have been previously ignored and SCL-PHA recovery methods have been applied to MCL-PHA as few methods have been available for the latter.

SCL-PHA recovery mainly involves the use of solvents to solubilize the PHA (Baptist, 1962; Lafferty and Heinzle, 1978; Vanlautem and Gilain, 1982) or enzymes, surfactants and/or inorganic chemicals to solubilize the non-PHA cellular materials (Holmes, 1984). With solvent extraction, high product purity and recovery efficiency have been achieved with MCL-PHA (Jiang et al., 2006). Solvents are commonly used in lab scale recovery. Safety concerns and high recycling costs make them less practical on a large scale. In non-solvent methods, surfactants have been used on various bacteria (Chen et al., 1999; Choi and Lee, 1999; DeKoning and Witholt, 1997; Dong and Sun, 2000), but they tend to adsorb to the PHA granules and are difficult to eliminate. Enzymes provide mild recovery conditions, but are expensive. For example, deKoning et al. (1997) estimated that the MCL-PHA recovery step was one third of the total

production cost, with almost half of the expense being enzymes and chemicals. Although hypochlorite was effective in PHB recovery (Berger et al., 1989), there was a molecular weight loss even at optimized digestion conditions (Ramsay et al., 1990). NaOH is easier to handle than solvents, less expensive than enzymes, milder than hypochlorite and has been used in PHB recovery (Choi and Lee, 1999). It was not clear whether it would be efficient in MCL-PHA recovery given the differences in SCL- and MCL-PHA properties. SCL-PHA is highly crystalline (Holmes, 1984) and denser than water (1.23 g/cm^3) (Nickerson, 1982), while MCL-PHA has a low degree of crystallinity (Marchessault et al., 1990; Gagnon et al., 1992) and a density close to that of water (1.019 g/cm^3) (Marchessault et al. 1995).

MCL-PHA recovery by NaOH digestion alone could be a cheap and simple method both at the bench scale and for industrial production. This study is the first to investigate the feasibility of MCL-PHA recovery based solely on NaOH digestion. The effect of NaOH concentration, temperature, contact time, and the number of treatment cycles were examined. An additional lysozyme treatment was evaluated to determine whether further purification could be obtained.

6.3 Materials and Methods

6.3.1 Microorganisms and chemicals

Pseudomonas putida KT2440 was cultured as reported previously with nonanoic acid as a single carbon source (Sun et al., 2007). The biomass was recovered by centrifugation at 6,000 ×*g* for 30 min, washed with distilled water, lyophilized and stored at -20^{o}C until used.

NaOH (ACS) and lysozyme (chicken egg white, 50000 units mg^{-1} protein) was purchased from Fisher Scientific and Sigma-Aldrich, respectively.

6.3.2 PHA recovery by NaOH digestion

6.3.2.1 Effect of NaOH concentration and temperature

Biomass samples (7% w/v) were mixed with 0 to 0.20 N NaOH at 22 $\pm 1^{o}$C from 5 min up to 4 h. Higher NaOH concentrations were not examined since there is a potential for PHA molecular weight loss. The mixture was vortexed for 15 sec at the lowest speed of a Fisher Vortex prior to sampling. Samples were recovered by centrifugation at 6,000 $\times g$ for 30 min and washed with distilled water. To study the effect of treatment temperature, the biomass was treated with 0, 0.05, 0.10, or 0.20 N NaOH as above, at 22, 40, 60 and 80 $\pm 1^{o}$C for 5 min.

6.3.2.2 Effect of two cycles of NaOH digestion

The two best conditions from the above experiments were combined in two cycles of NaOH digestion as shown in Table 6-1. Samples were recovered by centrifugation at 12,000 $\times g$ for 30 min and washed with distilled water.

Table 6-1 Treatment conditions of two-cycle NaOH digestion

Condition	1st cycle	2nd cycle
1	22°C, 0.2 N, 2 h	22°C, 0.2 N, 2 h
2	80°C, 0.1 N, 15 min	80°C, 0.1 N, 15 min
3	22°C, 0.2 N, 2 h	80°C, 0.1 N, 15 min

6.3.2.3 Lysozyme treatment after NaOH digestion

After two cycles of NaOH treatment (condition 3 in Table 6-1), the sample was further digested with 0.01 g lysozyme (50000 units mg protein^{-1}) g biomass^{-1} in 10 mM tris-HCl solution (pH 8.0) at 37°C for 1 h. Samples were recovered by centrifugation at 12,000 $\times g$ for 30 min and washed with distilled water.

6.3.3 Effect of digestion on molecular weight

Using a different sample of MCL-PHA with an initial molecular weight of 91.3 kDa, the effect of digestion on molecular weight was examined. The molecular weight of MCL-PHA was measured by gel permeation chromatography after biomass treatment at two levels of three independent variables (NaOH concentration, temperature, and treatment time) using a 2^3 factorial design (Table 6-2).

Table 6-2 High and low levels of three independent variables on the molecular weight of PHA in a factorial design experiment

Independent variable	Level	
	Low (-)	High (+)
A: Temperature	22 ^{o}C	80 ^{o}C
B: NaOH concentration	0.1 N	1 N
C: Digestion time	0.25 h	5h

6.3.4 Product analysis

6.3.4.1 PHA purity and recovery efficiency

A known amount of dried PHA sample was methylated with benzoic acid as the internal standard as described previously (Sun et al., 2007). Methylated samples (1 μL) were injected into a gas chromatograph (CP3900, Varian Inc.) equipped with a flame ionization detector. The injector and detector temperature were maintained at 250 and 275 ℃, respectively. The temperature-time profile was 90 ℃ for 0.5 min, 6 ℃ min^{-1} to 96 ℃, 7 ℃ min^{-1} to 131 ℃, 20 ℃ min^{-1} to 181 ℃, 181 ℃ for 5 min. A PHA standard was obtained by acetone extraction and methanol precipitation followed by purification (Jiang et al., 2006). A 10 mg standard sample was dissolved in 0.7 mL deuterated chloroform and its monomeric composition was determined by proton NMR with a Bruker AC-200

spectrometer. PHA purity was calculated as the percentage of the PHA mass of the total mass of the sample, while the recovery efficiency was based on the PHA mass recovered from a biomass of known PHA content.

6.3.4.2 Molecular weight

The weight average molecular weight was determined using a Waters 2695 gel permeation chromatograph with four Styragel columns of pore sizes 100, 500, 10^3,10^4 Å coupled to a Water 410 differential refractive index detector. The mobile phase was composed of distilled tetrahydrofuran with a flow rate of 1 ml min^{-1} at 40^{o}C. A calibration curve was obtained with eight polystyrene standards ranging from 870 to 355,000 Da. 10 mg standards and 50 mg samples were dissolved in 10 ml tetrahydrofuran for analysis.

6.4 Results

6.4.1 Effect of NaOH concentration as a function of time

Treatment of biomass with 0.01 N NaOH gave a similar PHA purity as untreated samples (Figure 6-1). Increasing the NaOH concentration from 0.01 to 0.20 N resulted in a linear increase in PHA purity after 5 min of digestion. The maximum purity after only 5 min of treatment was obtained with 0.20 N NaOH at 22 $\pm$ 1^{o}C, when PHA purity increased from 65.6 to 89.3%.

Generally, PHA purity increased as the digestion time increased, but very long treatment time (> 120 min) at higher NaOH concentrations (> 0.10 N) did not lead to further improvement. When the data in Figure 4-1 were re-plotted in an attempt to select the most cost efficient NaOH concentration, it can be seen that 0.10 N NaOH would be the best (Figure 6-2 (a) (b)). After 2 h of digestion in 0.20 N NaOH at 22 $\pm$ 1^{o}C, the PHA

molecular weight was unchanged at 101 kDa but decreased to 84 kDa after 4 h of digestion.

As the ratio of biomass to NaOH increased from 350 to 1400 g mol^{-1}, PHA purity decreased linearly at all treatment times (Figure 6-3). Longer treatment time up to 2 h at the same ratio resulted in higher purity. From the data provided, a set of digestion conditions (e.g. ratio of biomass to NaOH, and treatment time) can be selected to achieve a desired purity.

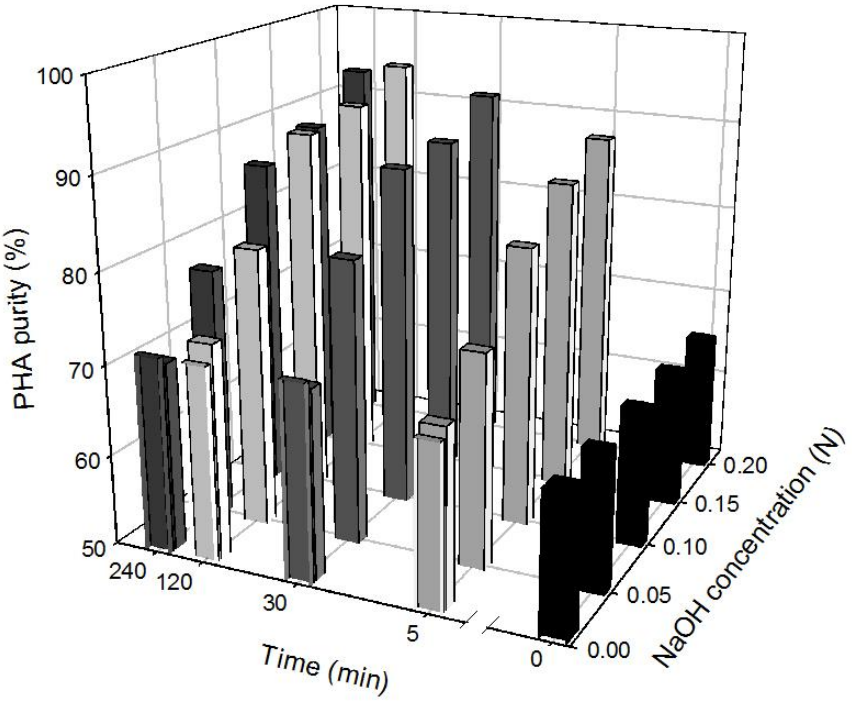

Figure 6-1 Effect of NaOH concentration on PHA purity as a function of treatment time at 22 ± 1°C.

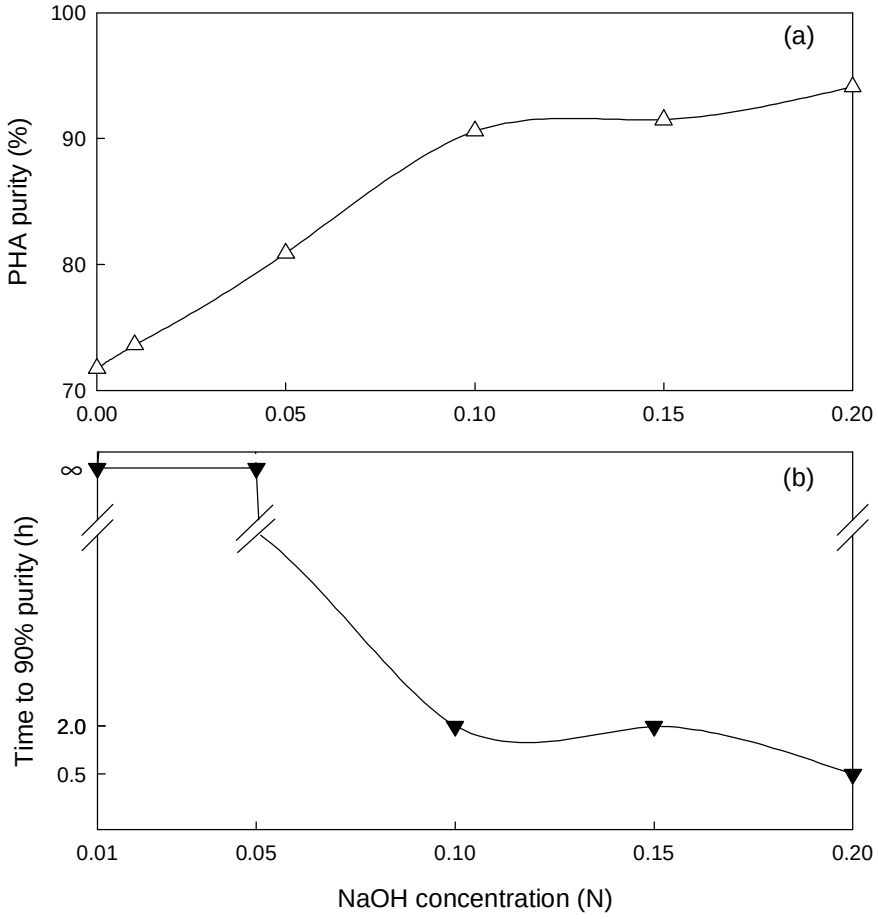

Figure 6-2 (a) Effect of NaOH concentration on PHA purity at 22 ± 1°C after 2 h of treatment; (b) time to achieve 90% purity at 22 ± 1°C (Data from Figure 6-1).

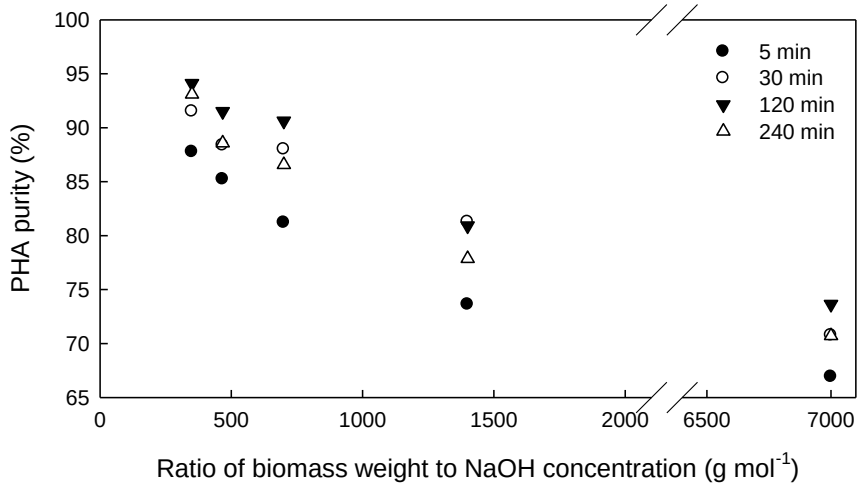

Figure 6-3 PHA purity with an increasing ratio of biomass (g) to NaOH concentration (mol^{-1}) at different treatment time (Data from Figure 6-1).

6.4.2 Effect of treatment temperature

PHA purity increased as the treatment temperature increased from 22 to 80^{o}C at all NaOH concentrations after 5 min of treatment (Figure 6-4). At 0.10 N NaOH, the recovered PHA had 93.7% purity at 80 $\pm$1^{o}C after 5 min of digestion. This purity is comparable to that obtained at 22 $\pm$1^{o}C after 2 h of digestion (Figure 6-1).

6.4.3 Two cycles of NaOH digestion

In the previous experiments the biomass samples had been vortexed in the digestion medium. This caused some of the biomass to adhere to the inside of the test tube outside of the digestion medium. In subsequent experiments, biomass samples were added to the NaOH solution and allowed to soak. Without mixing, a lower PHA purity was obtained indicating that mixing is important for efficient solubilization. However, mixing was not further studied.

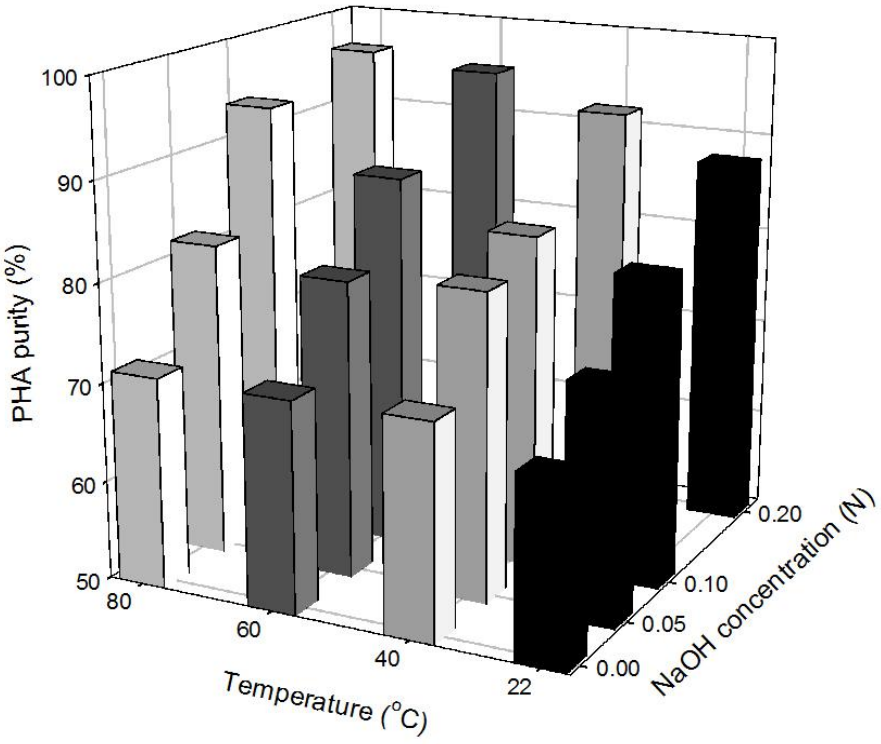

Figure 6-4 Effect of temperature on PHA purity at different NaOH concentrations after a 5 min treatment.

A preliminary experiment using two cycles of NaOH digestion at different temperatures eliminated a yellow color which remained after a single digestion of the biomass samples. Since this demonstrated a further improvement, we investigated the effect of two cycles of NaOH digestion at different temperatures on PHA purity and recovery (Table 6-3).

From an ANOVA analysis of the results (Table 6-4), any NaOH treatment significantly improved PHA purity ($p < 0.05$) when compared to the untreated biomass. A single cycle of NaOH digestion at 22 or 80 $\pm 1^{o}$C (Table 6-3) resulted in PHA purity of 88.7 $\pm$2.6% and 85.0 $\pm$5.3%, respectively. Sequential treatment at the same temperature

(22 or 80^{o}C) did not result in a significant improvement in PHA purity when compared to a single treatment at the same temperature ($p > 0.10$). However, sequential treatment at the two different temperatures achieved a significantly higher PHA purity than a single treatment at a confidence level of 90% ($p < 0.10$). The molecular weight of the recovered PHA (102 kDa with a polydispersity of 1.9) was unaffected as it was similar to the PHA from the untreated biomass (101 kDa with a polydispersity of 1.8).

An additional treatment with lysozyme did not significantly enhance purity at the 90% confidence level ($p > 0.10$) after sequential treatment at the two different temperatures.

6.4.4 Molecular weight loss

In a 2^3 factorial design experiment (Table 6-2), the effect of digestion conditions on PHA molecular weight was determined (Figure 6-5). Increasing the treatment temperature (22 to 80^{o}C) alone slightly decreased the molecular weight while increasing the NaOH concentration or treatment time alone decreased the molecular weight by 17 and 8%, respectively. However, a combination of increasing NaOH concentration and/or treatment time with increasing temperature significantly degraded the MCL-PHA polymer. A treatment at 80^{o}C in 1 N NaOH for 5 h resulted in a molecular weight of 351 Da which is close to the molecular weight of PHA oligomers.

Table 6-3 PHA purity and recovery efficiency after NaOH digestion

Trial	Treatment conditions[**]			PHA purity (%)	Recovery efficiency (%)
	1st cycle NaOH	2nd cycle NaOH	Additional digestion		
0	[*]–	–	–	65.6 ± 0.9[**]	–
1	22°C, 0.2 N, 2 h	–	–	88.7 ± 2.6	92.4 ± 2.3
2	80°C, 0.1 N, 15 min	–	–	85.0 ± 5.3	90.7 ± 1.4
3	22°C, 0.2 N, 2 h	22°C, 0.2 N, 2 h	–	87.9 ± 2.0	89.6 ± 1.7
4	80°C, 0.1 N, 15 min	80°C, 0.1 N, 15 min	–	91.5 ± 2.7	89.8 ± 2.2
5	22°C, 0.2 N, 2 h	80°C, 0.1 N, 15 min	–	94.7 ± 0.5	88.7 ± 1.0
6	22°C, 0.2 N, 2 h	80°C, 0.1 N, 15 min	0.01 g lysozyme g biomass^{-1}, 37°C, pH 7, 1 h	98.9 ± 2.7	83.6 ± 2.0

[*] Biomass sample was the un-treated control

[**] Error is reported as standard deviation of triplicate samples.

Table 6-4 Results of ANOVA analysis of data in Table 6-3 of NaOH treatment

Factors		T ratio	t_{crit}	Probability
Treatment vs. non-treatment	One cycle at 22°C vs. Control	14.56	4.30	0.0047
	One cycle at 80°C vs. Control	6.32	4.30	0.0242
	Two cycles at 22°C vs. Control	17.64	3.18	0.0004
	Two cycles at 80°C vs. Control	15.62	4.30	0.0041
	Two cycles at 22 and 80°C vs. Control	48.39	3.18	1.9E-05
Two-cycle vs. one-cycle	Two cycles at 22 °C vs. One cycle at 22°C	0.46	2.78	0.6686
	Two cycles at 80 °C vs. One cycle at 80 °C	1.90	3.18	0.1541
	Two cycles at 22 and 80 °C vs. One cycle at 22°C	3.94	4.30	0.0588
	Two cycles at 22 and 80 °C vs. One cycle at 80°C	3.18	4.30	0.0862
Non-enzymatic vs. enzymatic	Two cycles at 22 and 80 °C vs. Two cycles at 22 and 80 °C followed by lysozyme treatment	2.65	4.30	0.1181

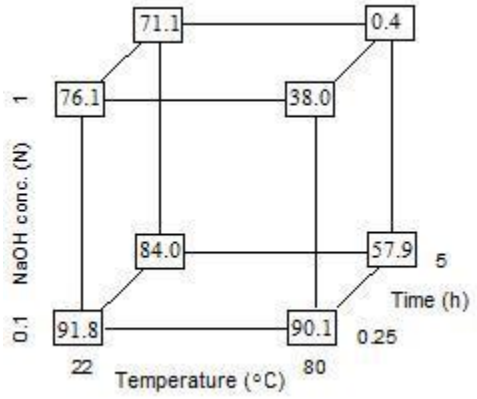

Figure 6-5 Effect of NaOH digestion on PHA molecular weight based on the factorial design shown in Table 4-2. The number in each box of the cube plot represents the average values of PHA molecular weight (kDa) from duplicate samples. The maximum error was ±1.1 kDa. PHA molecular weight in the biomass before treatment was 91.3 kDa.

6.5 Discussion

This is the first study to examine MCL-PHA recovery based on NaOH digestion of non-PHA cellular materials. A single cycle of digestion either in 0.20N at 22^{o}C for 2 h or in 0.1 N NaOH at 80^{o} for 15 min resulted in about 85% PHA purity from a biomass containing 65.6 ±0.9% MCL-PHA (Table 6-3). This suggests that a longer time at a lower temperature is required to achieve a similar purity as treatment at a higher temperature for a shorter time, probably because sufficient time is needed for the alkali to penetrate and to disintegrate the cell components. Two digestion cycles using a low followed by a high temperature (22^{o}C, 0.2 N NaOH for 2 h followed by 80^{o}C, 0.1 N NaOH for 15 min) achieved a higher PHA purity (94.7 ±0.5%) indicating that not all cellular materials were readily dissolved in NaOH at 22^{o}C when a PHA purity of 88.7 ± 2.6% was obtained after 2 h of treatment. It is important to note that there was no loss in PHA molecular weight under any of the above digestion conditions.

Additional lysozyme treatment seemed to enhance PHA purity, possibly by attacking peptidoglycan (DeKoning et al., 1997) but this improvement was not statistically significant.

MCL-PHA was more susceptible to molecular weight loss in an alkali environment at high temperature. Since treatment at high temperature and high NaOH concentration for a long time may lead to complete depolymerization, appropriate conditions must be selected to minimize this loss. The data presented in this paper will help in choosing such conditions. Molecular weight may be sacrificed to achieve certain benefits. For example, to remove endotoxins from PHB samples, Lee et al (1999) used NaOH digestion conditions (30^{o}C, 0.2 N NaOH for no less than 5 h or 30^{o}C, 2 N NaOH or higher for 2 h) which, based on our estimation, would probably decrease the molecular weight of a MCL-PHA from 91 to about 70 ~ 80 kDa.

Differences in the properties between SCL- and MCL-PHA would have affected the separation process. The lower density of MCL-PHA (1.019 g cm^{-3}) required the use of higher centrifugal force (6,000 ~12,000 $\times g$ 30 min) for recovery compared to 2,500 $\times g$ for 20 min to obtain the higher density SCL-PHA (1.23 g cm^{-3}). Using the lower centrifugal force would have resulted in a false low recovery. Furthermore an increase in alkaline hydrolysis is known to occur with PHB of higher crystallinity (Marchessault et al., 1994). Hence, it is likely that MCL-PHA (low crystallinity) would degrade less than SCL-PHAs (highly crystalline) under similar digestion conditions.

This work has demonstrated not only that NaOH digestion is a simple and efficient means of recovering MCL-PHA but it can be done under a wide range of conditions that do not significantly degrade the polymer. NaOH digestion also avoids the use of volatile

solvents, and establishes an MCL-PHA separation method that is easy to scaleup. The cost of chemicals would be lower compared to methods which required enzymes and other chemicals.

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Chapter 7

Conclusions

7.1 Summary and contributions

- MCL-PHA with an enriched content of the dominant monomer (e.g. 3-hydroxynonanoate (HN) or 3-hydroxyoctanoate (HO)) was produced by inhibiting β-oxidation with acrylic acid while co-feeding a carboxylic acid and a carbohydrate substrate. The co-feeding strategy allowed for efficient biomass production from glucose while the more expensive aliphatic substrate was almost entirely used for PHA production. Increasing acrylic acid concentration up to 0.2 g L^{-1} enabled a higher content of the dominant monomer, but had a negative effect on growth and PHA accumulation. For the first time, MCL-PHA containing 96 mol% HN or 98 mol% HO (almost homopolymeric) was produced efficiently in continuous cultivation. As the dominant monomer content increased the polymer's melting point, the degree of crystallinity, crystallization rate, tensile strength at break and Young's modulus increased.
- No previous study has developed an efficient fed-batch production of MCL-PHA with an enriched dominant monomer content. In this study, PHA containing up to 92 mol% HN was produced in a 7 L bioreactor by co-feeding nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25: 1: 0.05 and at a specific growth rate of 0.25 h^{-1}. Generally, high acrylic acid concentration in the feed produced a high HN percentage, but also led to an accumulation of toxic nonanoic acid and acrylic acid concentrations during exponential feeding. Lowering the bacterial

growth rate proved effective in achieving high biomass and PHA production efficiency without sacrificing a dominant monomer content. High biomass concentration, PHA content and cumulative PHA productivity (71.4 g L^{-1}, 75.5% and 1.8 g L^{-1} h^{-1}, respectively) could be attained at NA: G: AA= 1.25: 1: 0.05 and a specific growth rate of 0.15 h^{-1} with 88 mol% HN in the polymer. To maintain a desired level of inhibition, acrylic acid should be fed proportional to the carbon substrates to keep up with its consumption by the growing cells.

- This study was the first to examine MCL-PHA recovery conditions using acetone, a non-chlorinated solvent. A purified polymer was obtained following biomass pretreatment, acetone extraction and subsequent purification. Although NaOH was more effective in solubilizing cellular material other than PHA, both methanol and NaOH pretreatments gave the same PHA purity after acetone extraction. Soxhlet extraction was far more efficient than direct extraction, resulting a recovery of almost all PHA with 91.6% purity.
- NaOH digestion alone effectively recovered MCL-PHA from *Pseudomonas putida* KT2440. Factors influencing PHA purity included NaOH concentration, temperature, digestion time and the number of treatment cycles. Treatment at a lower temperature ($22 \pm 1^{o}C$) achieved similar PHA purity as at a higher temperature ($80 \pm 1^{o}C$) if a higher NaOH concentration or extended digestion time was used. Two cycles of NaOH solubilization (one at $22 \pm 1^{o}C$ followed by another at $80 \pm 1^{o}C$) was much more efficient than any single cycle digestion condition. Controlling all conditions was found to be essential to avoid molecular weight loss.

7.2 Recommendations for future work

Due to the comparatively high production cost, the current development of MCL-PHA applications undoubtedly aim at high value products in fields such as medicine and tissue engineering. Therefore, analytical methods need to be improved to accurately measure PHA purity and its impurities.

The production of MCL-PHA with enriched HN or HO in continuous cultivation and enriched HN in fed-batch fermentation indicates the possibility of producing PHAs with longer side-chains with a high content of the dominant monomer. Other β-oxidation inhibitors such as salicylic acid should be examined in the hope that a high amount of inhibition can be achieved with fewer harmful side effects.

To avoid early accumulation of toxic substrate(s) and consequent uncontrollable foaming, feeding strategies such as linear or a decaying feed after exponential feeding should be examined. These strategies may also help ease the oxygen demand late in the production phase when the oxygen transfer capacity in the bioreactor system is being approached.

A better understanding of the inhibition mechanism in β-oxidation may help to regulate the production of MCL-PHA with an enriched dominant monomer content in a more efficient way. Concentrations of key intermediates within the pathway should be analyzed and a metabolic model with more detailed metabolite synthesis rates should be constructed so as to probe the mechanism behind the experimental results.

Appendix A

Toxicity of acrylic acid on *Pseudomonas putida* KT2440

Materials and methods

A set of shake flask medium containing six concentrations of acrylic acid (0.0, 0.1, 0.2, 0.4, 0.8 and 2.0 g L^{-1}) received a 10% (v/v) inoculum and was incubated at 28.0 ±1.0^{o}C and 200 rpm. At 12 and 24 h, samples were taken and the optical density (OD) of each culture was measured at 650 nm. The medium for inoculum preparation and the toxicity test contained per liter: $(NH4)_2SO_4$ 4.70 g, $MgSO_4 \cdot 7 H_2O$ 0.80 g, $Na_2HPO_4 \cdot 7 H_2O$ 12.00 g, KH_2PO_4 2.70 g, nutrient broth 1.00 g, glucose 9.00 g.

Results

Cell growth decreased with increasing initial concentration of acrylic acid when *P. putida* was grown on glucose as the carbon and energy source in shake flasks (Figure A-1). An acrylic acid concentration of 0.2 g L^{-1} reduced the optical density two fold while there was very little growth at concentrations above 0.4 g L^{-1}.

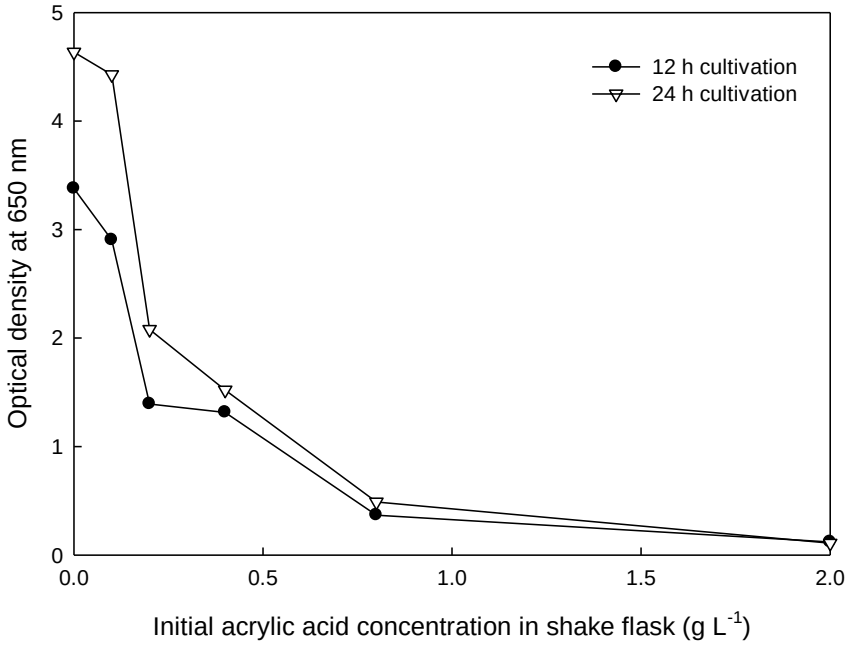


Figure A-1 Optical density of *P. putida* KT2440 grown in shake flasks with increasing initial acrylic acid concentration.

Appendix B

Metabolic flux analysis

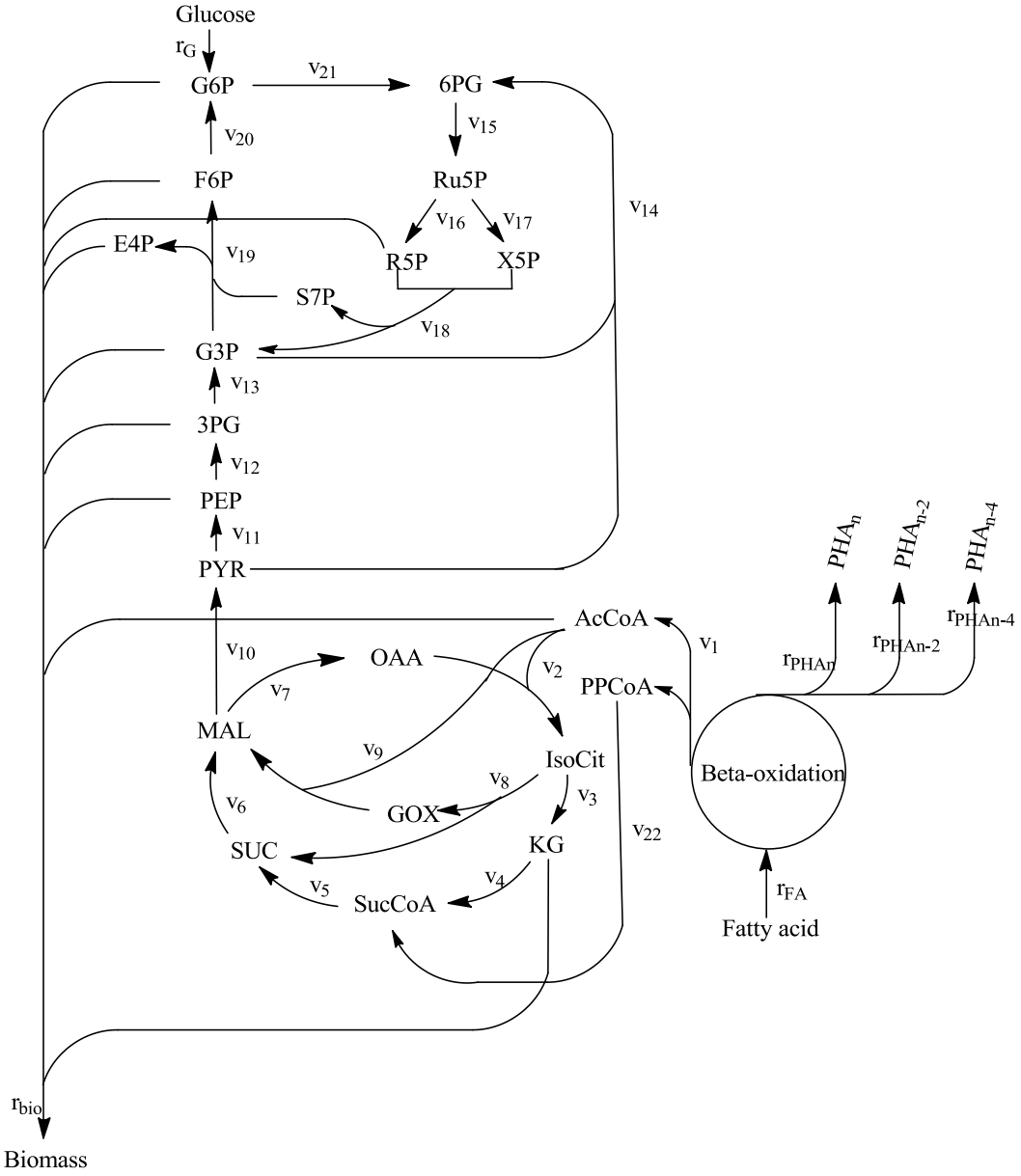
The metabolic flux analysis model in this appendix was developed by Bozhi Sun. Experimental work and data analysis were performed by the author.

Materials and methods

Pseudomonas putida KT2440 was cultivated as described in section 3.3 in Chapter 3. Data concerning the effect of acrylic acid on the production of biomass and PHA under single substrate (nonanoic acid) and co-substrate (nonanoic acid and glucose) feeding conditions were used in the model.

A stoichiometric model was setup based on the metabolic pathways illustrated in Figure B-1. To simplify, the pathways consisted of fatty acid utilization by β-oxidation, glucose utilization and associated PHA synthesis and biomass formation. The model assumed that NADPH and NADH are interchangeable and are expressed as NAD(P)H. Abbreviations in Figure B-1 were listed as below.

AcCoA	Acetyl co-enzyme A
ADP	Adenosine 5’ diphosphate
ATP	Adenosine 5’ triphosphate
CoA	Co-enzyme A
E4P	Erythrose-4-phosphate
FA	Fatty acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide, reduced
F6P	Fructose-6-phosphate
GOX	Glyoxylate
G3P	Glyceraldehyde 3-phosphate
G6P	Glucose-6-phosphate



$$\mathrm{NAD(P)H} + \frac{1}{2}\mathrm{O_2} \xrightarrow{v_{23}} \mathrm{NAD(P)} + 3\mathrm{ATP} + \mathrm{H_2O}$$

$$\mathrm{FADH_2} + \frac{1}{2}\mathrm{O_2} \xrightarrow{v_{24}} \mathrm{FAD} + 2\mathrm{ATP}$$

Figure B-1 Metabolic pathways of *P. putida* KT2440 grown on fatty acid and glucose as co-substrates. For the feeding of fatty acid alone, the pathways were the same except that no glucose was fed and the specific glucose consumption rate (r_G) was zero.

IsoCiT	Isocitrate
KG	Ketoglutarate
MAL	Malate
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NADH	Nicotinamide adenine dinucleotide (phosphate), reduced
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
3PG	3-phosphoglycerate
6PG	6-P-gluconate
PHA-n	PHA with n carbons, where n is the number of carbons in the fatty acid substrate
PHA-n-2	PHA with n-2 carbons
PHA-n-4	PHA with n-4 carbons
PYR	Pyruvate
r_{bio}	Residual biomass production rate (per gram of residual biomass, h^{-1})
r_{FA}	Fatty acid consumption rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
r_G	Glucose consumption rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
r_{PHA}	PHA synthetic rate (per gram of residual biomass, mmol g X_r^{-1} h^{-1})
Ru5P	Ribulose-5-phosphate
R5P	Ribose-5-phosphate
S7P	Sedoheptulose-7-phosphate
SUC	Succinate
SucCoA	Succinate co-enzyme A
X5P	Xylulose-5-phosphate

The balances of fluxes in Figure B-1 were as below.

1) $\mathrm{NA + CoA + 2ATP + 3FAD + 3NAD \rightarrow 3AcCoA + PPCoA + 3FADH_2 + 3NADH + 2ADP}$

2) $\mathrm{AcCoA + OAA + H_2O \rightarrow IsoCit + CoA}$

3) $\mathrm{IsoCit + NADP \rightarrow KG + NADPH + CO_2}$

4) $\mathrm{KG + CoA + NAD \rightarrow SucCoA + CO_2 + NADH}$

5) $SucCoA + ADP \rightarrow SUC + CoA + ATP$

6) $SUC + H_2O + FAD \rightarrow MAL + FADH$

7) $MAL + NAD \rightarrow OAA + NADH$

8) $IsoCit \rightarrow SUC + GOX$

9) $AcCoA + GOX + H_2O \rightarrow MAL + CoA$

10) $MAL + NADP + H_2O \rightarrow PYR + CO_2 + NADPH$

11) $PYR + 2ATP \rightarrow PEP + 2ADP$

12) $PEP \rightarrow 3PG$

13) $3PG + H_2O + ATP + NADH \rightarrow G3P + ADP + NAD$

14) $PYR + G3P \rightarrow 6PG$

15) $6PG \rightarrow Ru5P + NAD(P)H + CO_2$

16) $Ru5P \rightarrow R5P$

17) $Ru5P \rightarrow X5P$

18) $R5P + X5P \rightarrow G3P + S7P$

19) $G3P + S7P \rightarrow E4P + F6P$

20) $F6P \rightarrow G6P$

21) $6PG + NADPH \rightarrow G6P + NADP$

22) $PPCoA + CO_2 + ATP \rightarrow SucCoA + ADP$

23) $NAD(P)H + \frac{1}{2}O_2 \rightarrow NAD(P) + 3ATP + H_2O$

24) $FADH_2 + \frac{1}{2}O_2 \rightarrow FAD + 2ATP$

r_{bio}) $0.205\ G6P + 0.071\ F6P + 0.119\ G3P + 1.496\ 3PG + 0.519\ PEP + 2.833\ PYR +$
$3.747\ AcCoA + 1.079\ KG + 1.787\ OAA + 0.898\ R5P + 0.361\ E4P +$
$41.139\ ATP + 18.177\ NADPH \rightarrow biomass$

r_{FA}) $Fatty\ acid\ (extracellular) + 0.5ATP \rightarrow fatty\ acid\ (intracellular)$

r_G) $Glucose + ATP \rightarrow G6P + ADP$

$r_{PHA\text{-}n}$) $NA + CoA + 2ATP + FAD \rightarrow PHA - n + FADH_2 + 2ADP$

$r_{PHA\text{-}n\text{-}2}$)$NA + CoA + 2ATP + 2FAD + NAD \rightarrow PHA - n - 2 + 2FADH_2 + NADH +$
$2ADP + AcCoA$

$r_{PHA\text{-}n\text{-}4}$)$NA + CoA + 2ATP + 3FAD + 2NAD \rightarrow PHA - n - 4 + 3FADH_2 + 2NADH +$
$2ADP + 2AcCoA$

To build the model, the accumulation rate of intracellular metabolite, x_i, was defined as

$$x_i = \sum a_{ij} r_j \ (i = 1, 2, \cdots, m; j = 1, 2, \cdots, n)$$

Where a_{ij} is a stoichiometric co-efficient and r_j is the flux through reaction j. The matrix form of the model was expressed as

$$x = Ar$$

where x is an m-dimensional vector of metabolite accumulation rates, A is an m×n matrix of stoichiometric coefficients, and r is an n-dimensional vector of fluxes. Because the number of pathways is the same as the number of flux balances, the degree of freedom of vector r is 0. By determining substrate consumption rate (r_{FA}, r_G, mmol g X_r^{-1} h^{-1}), biomass production rate (r_{bio}, h^{-1}), PHA synthetic rate ($r_{PHA(n, n-2, n-4)}$, mmol g X_r^{-1} h^{-1}), other fluxes can be calculated. Since the carbon dioxide (CO_2) production rate was also recorded during the experiment, it was used to verify the calculation.

Results

Cultivation on nonanoic acid in the presence of acrylic acid

When nonanoic acid was the sole carbon source, *P. putida* KT2440 synthesized poly(3-hydroxynonanoate-co-3-hydroxyheptanoate) containing more than 68 mol% of HN (3-OH-nonanoate) with the use of acrylic acid at a concentration below 0.2 g L^{-1} (Figure 3-1, Chapter 3).

The flux v_1 represents the intermediate flow leaving from β-oxidation. It was assumed, in this model, to be the only route connecting fatty acid utilization with the rest of the metabolic pathways. Unlike what was expected as the inhibition became intense,

this flux showed a trend of increase (Figure B-2), indicating the existence of other metabolic routs which was able to break down fatty acids.

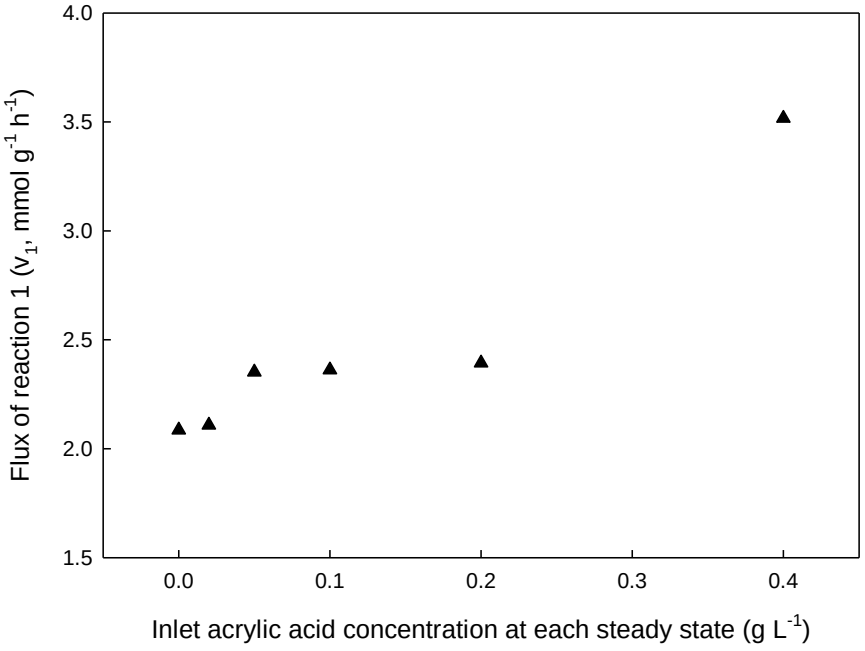


Figure B-2 Simulated flux of reaction 1 (v_1) as increasing inlet acrylic acid concentration when *P.putida* KT2440 was grown on nonanoic acid in continuous cultivation.

Since nonanoic acid was the only carbon substrate, the profile of the simulated CO_2 production rate was almost the same as that of flux v_1 but on different scales (Figure B-3 (a)). It suggested more energy consumption on respiration as increasing the inlet acrylic acid concentration. The clearly high values of CO_2 production rate (Figure B-3 (a)) and flux v_1 (Figure B-2) at 0.4 g L^{-1} acrylic acid compared to those below 0.2 g L^{-1} acrylic acid correlated with the fermentation results with no PHA accumulation and obviously lower biomass concentration (Figure 3-1, Chapter 3). The residues between the simulated and experimental CO_2 production rate showed fairly distributed errors (Figure B-3 (b)).

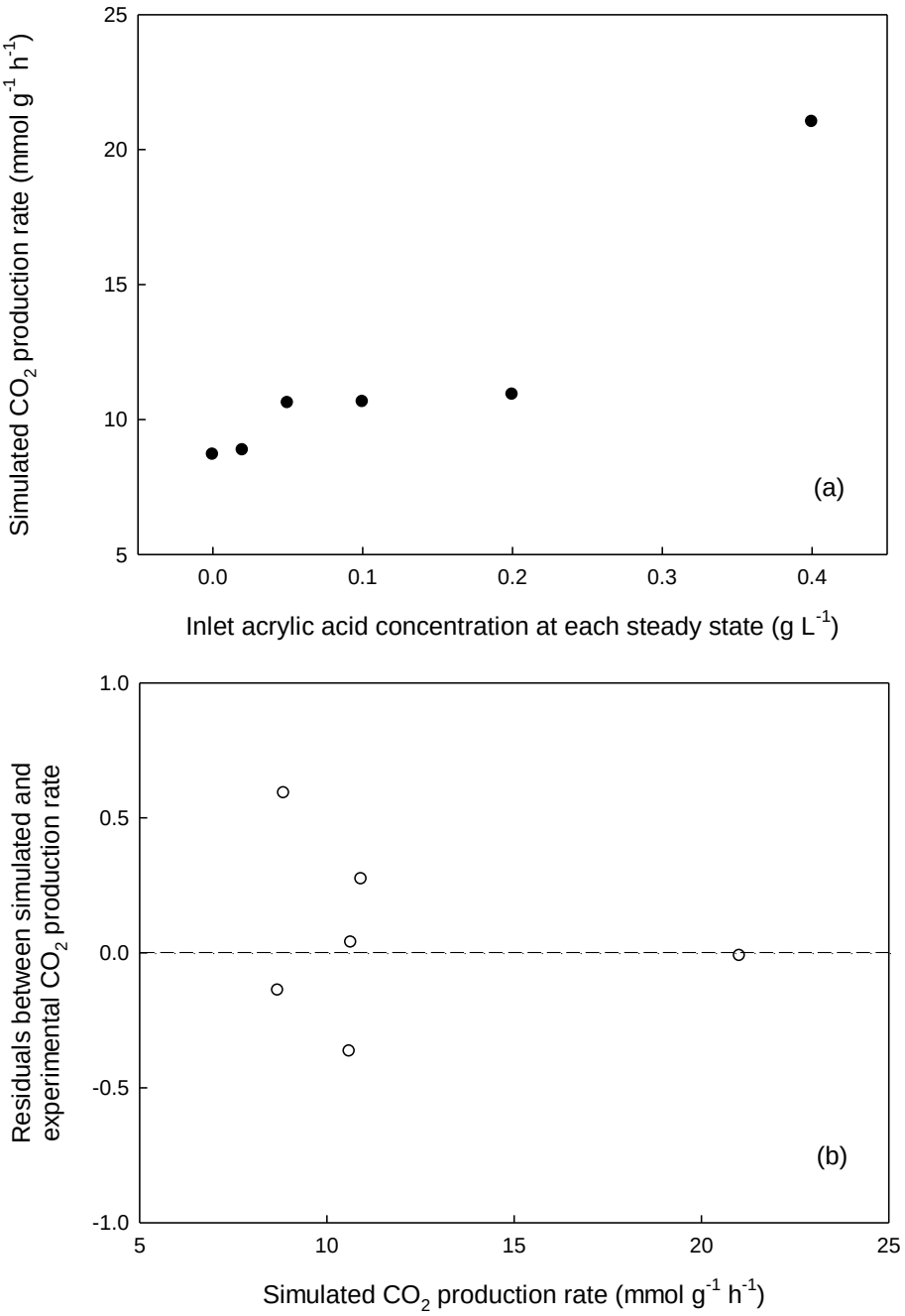


Figure B-3 Simulated CO_2 production rate as increasing the inlet acrylic acid concentration when *P. putida* KT2440 was grown on nonanoic acid in continuous cultivation.

Cultivation on nonanoic acid and glucose at the presence of acrylic acid

When nonanoic acid and glucose were fed as co-substrates, the flux of v_1 has a V-type profile (Figure B-4). It suggested that when the inlet acrylic acid concentration was lower than 0.15 g L^{-1}, the effect of β-inhibition was dominant. When the inhibitor was higher, some inhibitor effect showed and the fatty acid metabolism was adjusted physiologically. This unclear effect corresponds to the production phenomenon where the HN monomer percentage increased to 95 mol% and decreased slightly thereafter (Figure 3-2, Chapter 3).

The CO_2 production rate increased gradually from 8 to 14 mmol g^{-1} h^{-1} (Figure B-5 (a)) with the increase of inlet acrylic acid concentration from 0 to 0.15 g L^{-1}. The increase was a little faster compared to that under nonanoic acid single feeding in Figure B-3 (a), probably due to the readily availability of glucose as energy source under fatty acid inhibition conditions. However, the CO_2 production rate curve was relatively stable when the acrylic acid concentration was higher, indicating cell tolerance on the inhibitor due to the carbohydrate co-feeding. And this corresponded to the fermentation outcomes that there were only slight changes in biomass concentration and PHA content when acrylic acid concentration changed from 0.15 to 0.45 g L^{-1}. The residual plot of CO_2 production rate showed fair error distribution (Figure B-5 (b)).

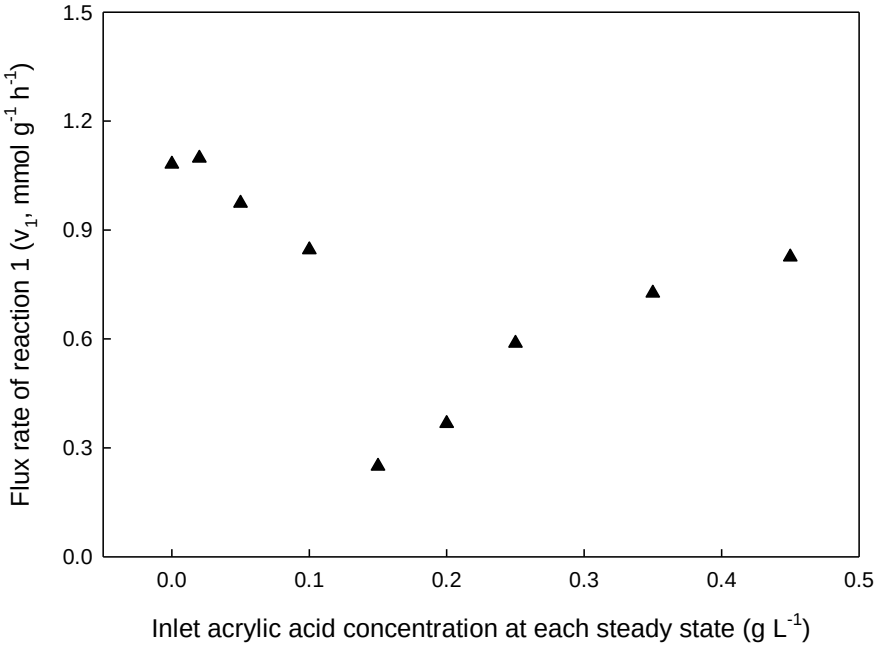


Figure B-4 Simulated flux of reaction 1 (v_1) as increasing inlet acrylic acid concentration when *P. putida* KT2440 was grown on nonanoic acid and glucose in continuous cultivation.

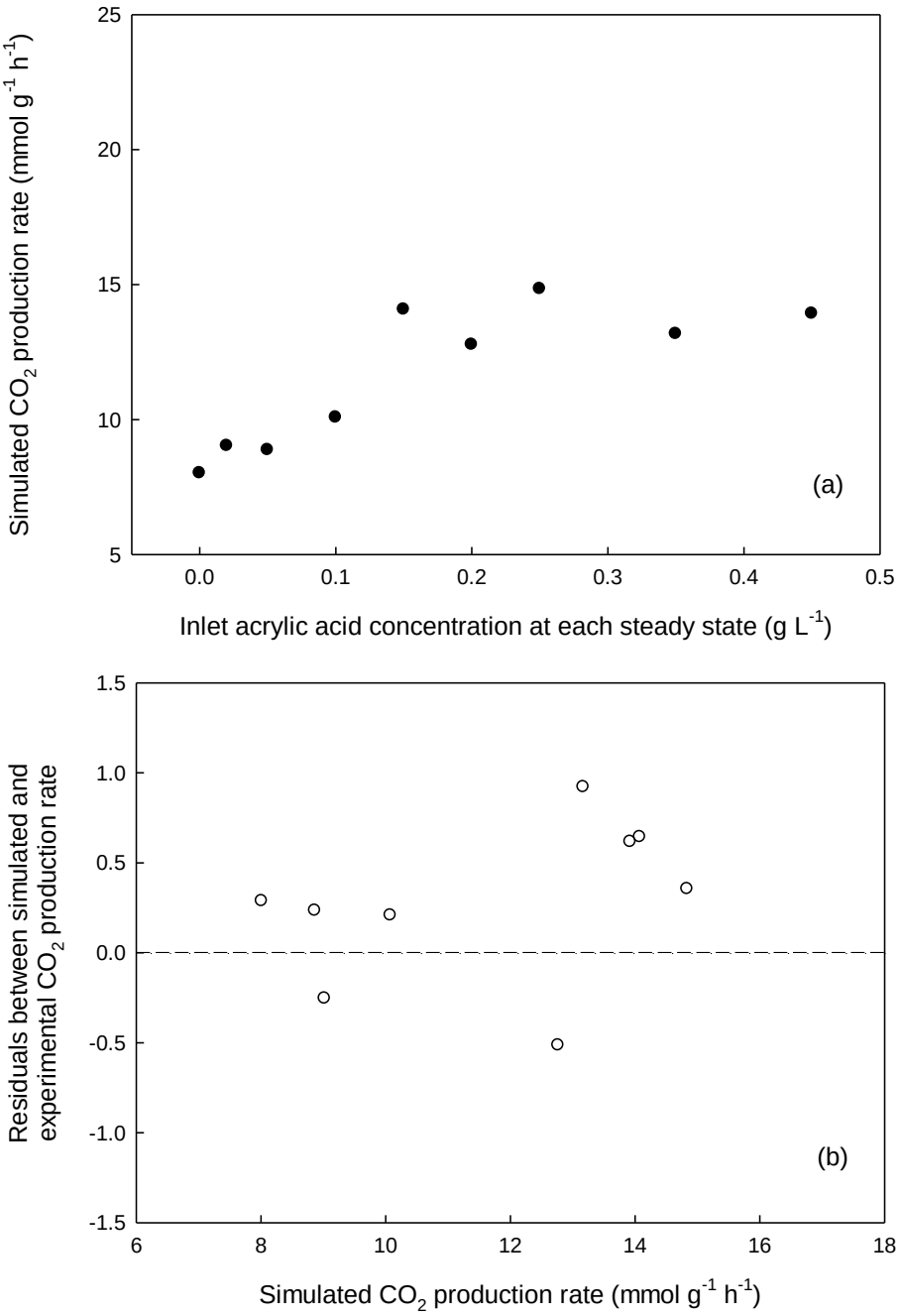


Figure B-5 Simulated CO_2 production rate as increasing the inlet acrylic acid concentration when *P. putida* KT2440 was grown on nonanoic acid and glucose in continuous cultivation.

Appendix C

Thermal degradation of MCL-PHA in acetone, chloroform and tetrahydrofuran

Materials and methods

MCL-PHA, containing 70 mol% of 3-hydroxynonanoate and 30 mol% of 3-hydroxyheptanoate, was extracted with acetone and had a purity of above 99%. Three sets of samples, seven for each set, were prepared separately with acetone, chloroform and tetrahydrofuran. Each sample contained 0.05 g PHA in 10 mL solvent, sealed and placed in an oven at 73 ± 2^{o}C. By the end of day 3, 7, 10, 14, 21, 28, one sample from each set was taken and the solvent was evaporated under vacuum. Dried sample residuals were dissolved in 10 mL distilled tetrahydrofuran and analyzed for their molecular weights. All solvents were at HPLC grade. Acetone and chloroform were purchased from Sigma-Aldrich, and tetrahydrofuran from B & J Brand®.

Results

For MCL-PHA dissolved in either acetone or tetrahydrofuran, there was no significant molecular weight change within the first two weeks (Figure C-1). However, PHA degradation in chloroform started from the very beginning and the molecular weight went down continuously to one fifth of its original value by day 14.

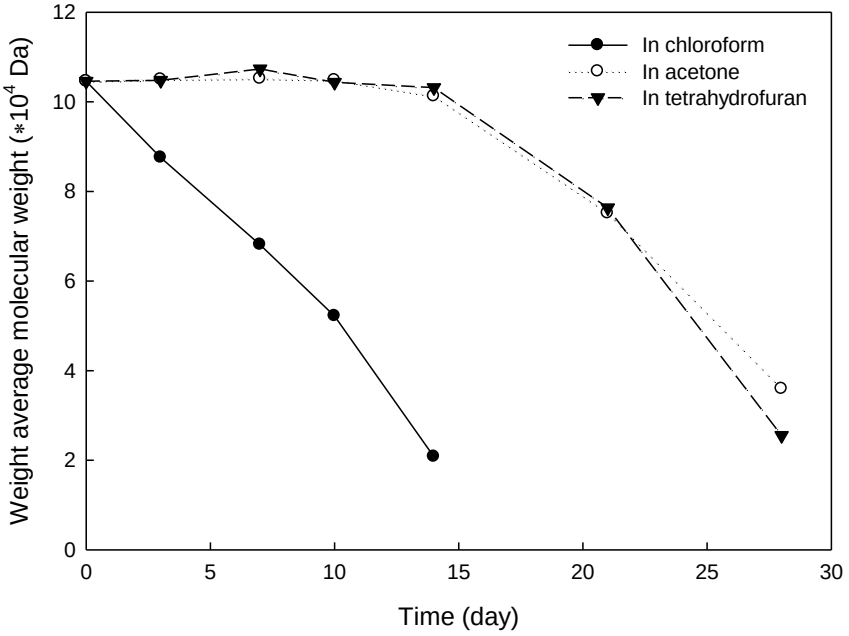


Figure C-1 Effects of solvents on PHA molecular weight at 73 $\pm 2^{o}$C.

Appendix D

Determination of impurities in MCL-PHA samples

Materials and methods

Nitrogen analysis

The nitrogen content of the samples in Table D-1 was analyzed by total Kjeldahl nitrogen (TKN) and elemental analyses.

Table D-1 Biomass and PHA samples analyzed by total Kjeldahl nitrogen and elemental analyses

No.	% PHA	Carbon sources in fermentation	Treatment	Reference
1	10	Glucose, nonanoic acid	No treatment	Sun et al., 2007a
2	46	Glucose, nonanoic acid	No treatment	Sun et al., 2009
3	62	Nonanoic acid	No treatment	Sun et al., 2007b
4	74	Nonanoic acid	NaOH digestion	Chapter 6
5	81	Nonanoic acid	NaOH digestion	Chapter 6
6	96	Nonanoic acid	NaOH digestion	Chapter 6

In TKN analysis (APHA, 1971), a 0.08 – 0.4 g sample was digested in 7 mL hot concentrated sulfuric acid with 2 g potassium persulphate, cooled then diluted to 100 ml with deionized water. After that, 25 ml of each sample was neutralized with 25% (w/v) NaOH using four drops of Methyl Red indicator in a 125 ml Erlenmeyer flask. Sodium-EDTA solution (4%, w/v) was added to remove interfering iron and calcium ions. After filtering with glass filter paper, the solution was back titrated with 10% (v/v) sulfuric acid with 3 drops in excess to a pinkish red color, diluted to 100 ml again then analyzed at 630 nm in a Gilson Auto colorimeter. The analysis was performed by Caduceon Labs, Kingston, Ontario.

Nitrogen and carbon were measured using a Costech Elemental Analyser ECS 4010 coupled to a Finnigan MAT Delta Plus XP Stable Isotope Ratio Mass Spectrometer operating in continuous flow mode. The analysis was performed by the Stable Isotope Lab, Department of Geological Sciences, Queen's University, Kingston, Ontario.

Muramic acid analysis

After the samples were pretreated to remove proteins and other metal interfering substances according to Van Hee et al. (2004), they were hydrolyzed at 90-100^{o}C in 96% sulfuric acid for at least 30 min, during which muramic acid was converted to lactic acid then to acetaldehyde (Taylor, 1996). After cooling to room temperature, a 3.5 mL sample was mixed with 50 µL 4% (w/w) copper sulphate pentahydrate and 100 µL 1.5% (w/w) *p*-phenylphenol in 95% ethanol. The absorbance was measured at 570 nm using a lactic acid calibration curve from 29.5 μg to 177 μg of a 1.18 μg/μL standard lactic acid solution (Figure D-1).

Results

Nitrogen impurity

Nitrogen is a major element in cell components but not in PHA. A typical bacterial cell contains 12% nitrogen by dry weight (Madigan et al., 2000). TKN and elemental analyses showed that *Pseudomonas putida* cells in this study contained less than 6% of nitrogen and demonstrated a similar trend (Figure D-2) in which the nitrogen content decreased as the PHA content increased.

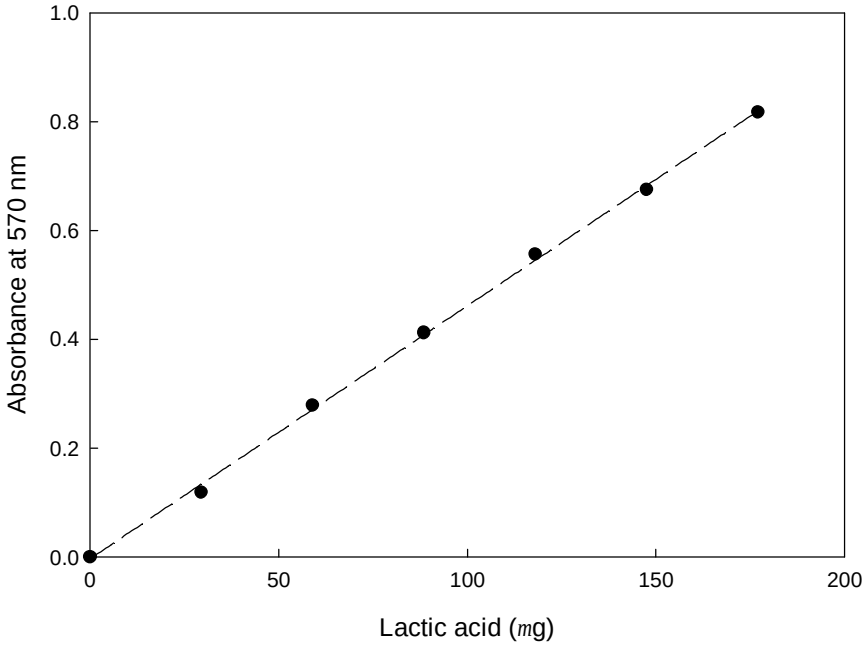


Figure D-1 Calibration curve of lactic acid at 570 nm.

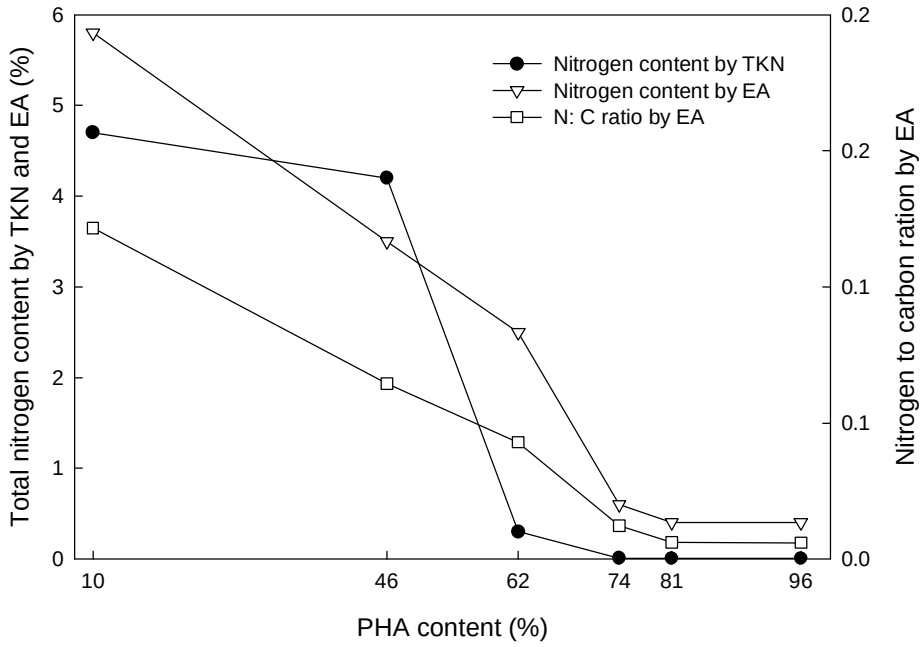


Figure D-2 Nitrogen analysis of samples with different PHA content. EA represents elemental analysis, while TKN represents Total Kjeldahl Nitrogen analysis.

The nitrogen to carbon ratio also decreased as the PHA content increased. A desirable nitrogen to carbon ratio is ultimately zero, indicating the complete removal of all nitrogen-containing cellular components. The nitrogen to carbon ratio of a PHA sample with 96% purity was 0.006 in this experiment.

The error associated with the elemental analysis is ±1%, while the error with TKN is up to 4% depending on the nitrogen concentration in the sample (data provided by Caduceon Labs, Kingston, Ontario). In Figure D-2, the TKN values were extremely low at high PHA content, and likely causes an underestimation of the nitrogen content due to its lower sensitivity on solid samples with low nitrogen levels.

Muramic acid as an indicator of residual peptidoglycan

Peptidoglycan is a major component of the bacterial cell wall. The elimination of peptidoglycan can be a good indicator of the removal of all cell debris. In Table D-2, as PHA purity increased, muramic acid decreased, indicating partial disintegration of peptidoglycan during PHA recovery.

Table D-2 Muramic acid in PHA samples treated by NaOH digestion

Samples	PHA purity (%)	Muramic acid content (%)
0	65.6 ±0.9	1.14 ±0.03
1	88.7 ±2.6	0.76 ±0.02
2	85.0 ±5.3	0.55 ±0.15
3	87.9 ±2.0	0.48 ±0.06
4	91.5 ±2.7	0.38 ±0.10
5	94.7 ±0.7	0.41 ± 0.07
6	98.9 ± 2.7	0.28 ± 0.04

Errors were calculated as the standard deviations of triplicate samples.

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