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Production of short-chain-length/medium-chain-length polyhydroxyalkanoate copolymers in *Cupriavidus necator*

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DEDICATION

I dedicate this Dissertation to my father Rathinasabapathy and my mother Shanthi Rathinasabapathy whose love, ardent enthusiasm and moral support has been the pillars for building this project. My special salutation to Aunty Saroja Radhakrishnan for her affectionate blessing. I also dedicate my thesis to twin Brothers: Thirunavukkarasu & Thirugnanasambandham; Sister Mangayarkarasi Saravanan with junior Anirudh for their constant encouragement and sacrifice they endured for the successful completion of my work.

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In my childhood days, I used to learn by heart "*Thirukkural*" (*El* '*Coplas sagradas' es una obra célebre, elogiada como código de conducta ética para la humanidad*) on a daily basis. The evergreen couplet I ever remember *in Tamil* is:

கற்க கசடறக் கற்பவை கற்றபின்

நிற்க அதற்குத் தக.

In Spanish: "Aprende perfectamente todo lo que aprendas, y después mantén tu conducta digna de lo que aprendiste". *In Englsih*: "So learn that you may full and faultless learning gain, then in obedience meet to lessons learnt remain". The explanation is "*Let a man learn thoroughly whatever he may learn, and let his conduct be worthy of his learning"*. Thereby I acknowledge the author *Thiruvalluvar* because *the essence of the couplet reached me in depth and assisted me all through the journey of my education and this became the limelight to do my doctoral studies*.

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ABBREVIATIONS AND ACRONYMS

CINVESTAV	Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional
CONACYT	National Council of Science and Technology
NSERC	Natural Science and Engineering Research Council
PHA/3HA	Polyhydroxyalkanoate/3-hydroxyalkanoate
PHB/3HB/C4	Polyhydroxybutyric acid/3-hydroxybutyrate
PHV/3HV/C5	Polyhydroxyvaleric acid/3-hydroxyvalerate
3HHx/C6	3-hydroxyhexanoate
3HHp/C7	3-hydroxyheptanoate
3HO/C8	3-hydroxyoctanoate
3HN/C9	3-hydroxynonanoate
3HD/C10	3-hydroxydecanoate
3HDD/C12	3-hydroxydodecanoate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-4HB)	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
P(3HB-co-3HHx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
scl	Short-chain-length
mcl	Medium-chain-length
HCDC	High-cell-density cultivation
O	Oxygen
N	Nitrogen
P	Phosphorus
S	Sulphur
Mg	Magnesium
K	Potassium
C/N	Carbon to nitrogen ratio
DO	Dissolved oxygen expressed as percentage of air saturation, %
μ	Specific growth rate, (h^{-1})
X	Biomass, (g/l)
X_r	Residual biomass (X minus PHA concentration), (g/l)
$Y_{X/S}$	Yield of biomass from substrate, (g/g)
CPR	Carbon dioxide production rate (g/h)
T_m	Melting temperature (°C)
T_g	Glass transition temperature (°C)

RESUMEN

En el presente trabajo se presenta una estrategia de alimentación desarrollada para mejorar la incorporación de monómeros de cadena media, derivados del aceite de canola, al polihidroxialcanoato copolímero de cadena corta y media (scl-mcl PHA) sintetizado por *Cupriavidus necator*. Para ello se utilizó un proceso de alimentación por lote alimentado y una modificación genética de *Cupriavidus necator*. Posteriormente, se evaluaron las propiedades mecánicas de los copolímeros producidos tanto por la cepa silvestre, como por la cepa transformada. En la primera fase de este estudio se produjo por fermentación, una alta concentración de biomasa de la cepa silvestre *Cupriavidus necator* H16, crecida con fructosa para producir suficiente copolímero de cadena corta (scl), así como polihidroxialcanato de cadena media (mcl) empleando aceite de canola. De esta manera se obtuvo suficiente copolímero para realizar su caracterización mecánica. Se inició con cultivo por lote seguido de una alimentación exponencial de fructosa a una μ predeterminada hasta alcanzar 44.4 g de biomasa/l, conteniendo solo 20 % w/w de polihidroxibutirato (PHB) con una $Y_{x/fructosa}$ de 0.44 g/g. Posteriormente se agregó aceite de canola bajo condiciones de limitación de N para producir 92 g/l de biomasa con 48 % w/w de scl-mcl de PHA. Utilizando estándares conocidos, se confirmó la composición del PHA por GC-MS obteniéndose un 99.81 % de 3-hidroxibutirato (3HB), 0.06 % de 3–hidroxivalerato (3HV), 0.09 % de 3-hidroxihexanoato (3HHx), y 0.04 % de 3-hidroxioctanoato (3HO). Los resultados para el estándar de PHB fueron: temperatura de fusión (179ºC), cristalinidad (54 %), esfuerzo a la tensión (25.1Mpa) y módulo de Young (698 Mpa), mismos que disminuyeron a 176ºC, 52 %, 19.1 Mpa y 443 Mpa respectivamente para el PHA de *C. necator* producido en el proceso de tres etapas.

Para la segunda fase de este estudio se realizó la coexpresión de la acyl-CoA deshidrogenasa (*fadE*$_{Ec}$) de *Escherichia coli* y la enoyl-CoA hidratasa (*phaJ1*$_{Pp}$) de *Pseudomonas putida* en *Cupriavidus necator* pMPJAS03, utilizando como promotor *araC*-P_{BAD} con la finalidad de incrementar la incorporación de monómeros mcl en el copolímero de scl-mcl PHA. La única diferencia con el proceso de trea etapas para producir scl-mcl PHA con la cepa transformante, fue la adición de arabinosa para inducir el promotor *araC*-$P_{BAD,}$ mejorando la acumulación de mcl PHA a partir del aceite de canola bajo limitación de N. Conforme se incrementó la concentración de inductor de 0 a 0.1 %, la biomasa y la producción de PHA disminuyeron. La composición del monómero de PHA se confirmó por análisis GC-MS, siendo 3-hidroxibutirato (3HB), 3-hidroxivalerato (3HV), 3-hidroxihexanoato (3HHx), 3-hidroxioctanoato (3HO) y 3-hidroxidecanoato (3HD), donde los monómeros diferentes a 3HB variaron el rango de 0.20 a 0.44 mol %. Cuando se comparó con el estándar de PHB, la temperatura de fusión (179ºC) y la cristalinidad (54 %) disminuyeron sensiblemente, alcanzando valores de 169ºC y 45 % respectivamente para el copolímero de *C. necator* pMPJAS03. Los valores del módulo de Young y el esfuerzo a la tensión disminuyeron conforme la cantidad de monómeros diferentes al 3HB se incrementó.

ABSTRACT

A feeding strategy for the improved incorporation of canola derived medium-chain-length monomers into short-chain-length/medium-chain-length polyhydroxyalkanoate copolymers (scl-mcl PHA) was developed employing fed-batch fermentation process and genetic modification of *Cupriavidus necator*. Further, the mechanical properties of the scl-mcl PHA copolymer produced by the wildtype and the transformant were evaluated. For the first approach in this study, wildtype *Cupriavidus necator* H16 biomass was grown on fructose to produce sufficient copolymer of short-chain-length (scl) and medium-chain-length (mcl) polyhydroxyalkanoate (PHA) from canola oil for mechanical testing of the PHA. Initial batch cultivation on fructose was followed by exponential feeding of fructose at a predetermined μ to achieve 44.4 g biomass/l containing only 20 % w/w of polyhydroxybutyrate (PHB) with a $Y_{x/fructose}$ of 0.44 g/g. In a third stage, canola oil was added under N-limited conditions to produce 92 g/l of biomass with 48 % w/w scl-mcl PHA. Using known standards, the PHA composition was confirmed by GC-MS analysis as 99.81 % 3-hydroxybutyrate (3HB), 0.06 % 3-hydroxyvalerate (3HV), 0.09 % 3-hydroxyhexanoate (3HHx) and 0.04 % 3-hydroxyoctanoate (3HO). The melting temperature (179°C), crystallinity (54 %), tensile stress (25.1 Mpa) and Young's modulus (698 Mpa) for a PHB standard decreased to 176°C, 52 %, 19.1 Mpa and 443 Mpa respectively for *C. necator* PHA produced in the 3-stage process.

In the second approach, co-expression of acyl-CoA dehydrogenase ($fadE_{Ec}$) from *Escherichia coli* and enoyl-CoA hydratase ($phaJ1_{Pp}$) from *Pseudomonas putida* in *Cupriavidus necator* pMPJAS03, under an *araC*-P_{BAD} promoter system, was used in an attempt to enhance the proportion of mcl monomers in the scl-mcl PHA copolymer than the wildtype. The only difference in the 3-stage process to produce scl-mcl PHA with the transformant was the addition of inducer – arabinose, to induce the *araC*-P_{BAD} promoter system enhancing the mcl PHA accumulation from canola oil under N-limitation. As the inducer concentration was increased

from 0 to 0.1 %, biomass and PHA production decreased. The monomer composition of PHA was confirmed by GC-MS analysis as 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) where the monomers other than 3HB totalled 0.20 to 0.44 mol %. When compared to a PHB standard, the melting temperature (179°C) and crystallinity (54 %) decreased to 169°C and 45 % for *C. necator* pMPJAS03's copolymer. The Young's modulus and tensile stress values decreased as the amount of monomers other than 3HB increased.

1 Introduction

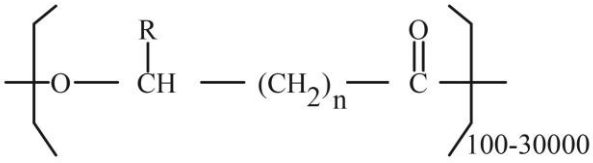
1.1 Plastic – an indispensable material

Plastics are common materials used in our daily lives. Synthetic plastics are produced from petroleum and their advantages include being easily molded into complex shapes, highly resistant to chemicals and elastic in nature to make them popular packaging materials. Although these materials are highly useful, their non-degradable nature poses a threat to the environment. They accumulate in the natural and artificial ecosystems, creating harm to flora and fauna. In the current scenario, complete abolition of plastic usage is impossible but substitution with biodegradable alternatives with similar characteristics as synthetic plastics and being made from renewable resources is of commercial interest. Commercially produced biodegradable polymers include polyhydroxyalkanoates (PHAs), polylactides (PLAs), aliphatic polyesters, polysaccharides and blends of starch (Vroman and Tighzert 2009). Although PLA is widely used, their applications are limited because they crystallize slowly, have poor heat resistance and are mechanically brittle (Yokohara and Yamaguchi 2008). Starch blends are considered as semi-biodegradable whereas PHAs are 100 % biodegradable (Patwardhan and Srivastava 2004). Therefore, among the biodegradable polymers, PHAs are of potential interest not only because they are completely biodegradable but also they have material properties similar to conventional plastics.

1.2 Polyhydroxyalkanoates

PHAs are macromolecules composed of 3-, 4-, 5- and 6- hydroxycarboxylic acids in which the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. PHA was first discovered by Maurice Lemoigne in 1926 with the exploration of polyhydroxybutyrate (PHB) synthesized from the soil bacterium *Bacillus* (Madison and Huisman 1999). The functional role of PHB as intracellular reserve granule was first demonstrated by Macrae and Wilkinson in 1958 (Madison and Huisman 1999). At present, more than 250 different bacteria including Gram negative and Gram positive bacteria have been shown to synthesize PHA as intracellular reserve granules when carbon source is in excess.

1.2.1 Chemical structure



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

Figure 1.1: Chemical structure of polyhydroxyalkanoates (Ojumu et al. 2004)

The general structure of PHA is shown in Figure 1.1. PHAs are polymers composed of carbon, hydrogen and oxygen. The hydroxyl substituted carbon atoms are of R-configuration, though some exceptions occur. In the β position, R refers to the length of the side chain and n refers to the size of the alkyl group. Therefore R and n together determines the type of hydroxyalkanoate (HA) monomer unit (Loo and Sudesh 2007; Ojumu et al. 2004). The most characterized PHAs have side chain varying between C1 to C14 at the C3 position. Over 155 different constituents of PHAs with unique monomer composition has been reported with some examples shown in Figure 1.2 (Agnew and Pfleger 2012; Steinbüchel and Lütke-Eversloh 2003; Steinbüchel and Valentin 1995). The broad range of monomers other than alkyl groups are 3-hydroxy acids with unsaturated, branched, substituted side chain, aromatic side groups, as well as non 3-hydroxy acids such as 4-hydroxyvaleric acid and 5-hydroxyhexanoic acid have been reported (Figure 1.3) (Rai et al. 2011). These also include dimethyl substituted carbons, terminal methyl and fluorophenoxy, thiophenoxy, oxo (keto) and acetylthioester groups (Agnew and Pfleger 2012)

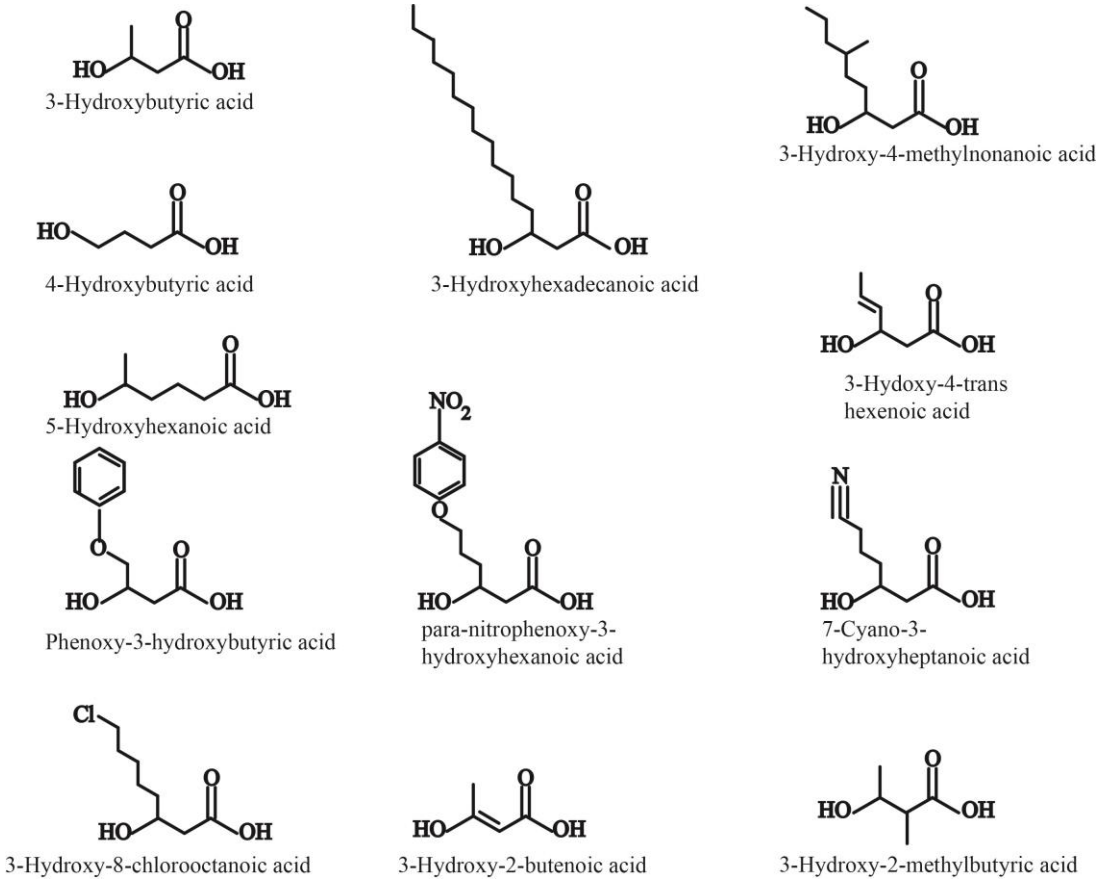


Figure 1.2: Some examples of constituents of biosynthetic PHAs (Steinbüchel 2001)

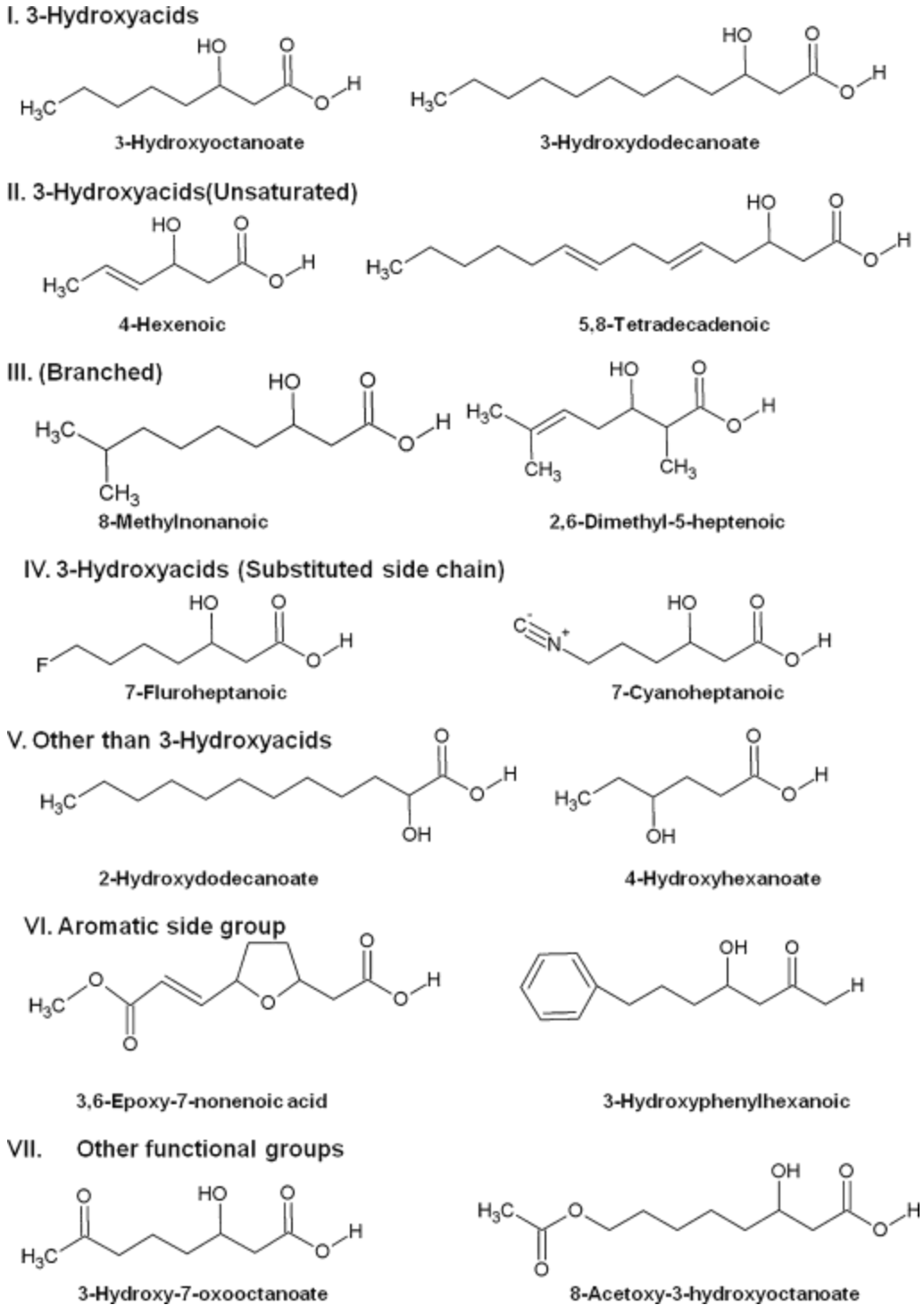


Figure 1.3: A wide range of monomers found in mcl PHAs (Rai et al. 2011)

1.2.2 Classification

PHAs can be divided into three broad categories based on the number of carbons atoms in the 3-hydroxyl fatty acid monomers: short-chain-length (scl) PHAs contain up to five carbons, medium-chain-length (mcl) PHAs have 6-14 carbons and long-chain-length (lcl) PHAs have more than 14 carbons (Figure 1.4) (Madison and Huisman 1999). Typical examples of scl PHA units are poly(3-hydroxybutyrate), PHB; poly(3-hydroxyvalerate), PHV; and poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHBV copolymer and examples of mcl PHAs are poly(3-hydroxyoctanoate), PHO; and poly(3-hydroxynonanoate), PHN; that are mainly formed as copolymers of 3-hydroxyoctanoate or 3-hydroxynonanoate together with 3-hydroxyhexanoate, 3HHx; 3-hydroxyheptanoate, 3HP; and/or 3-hydroxydecanoate, 3HD (Hazer and Steinbüchel 2007).

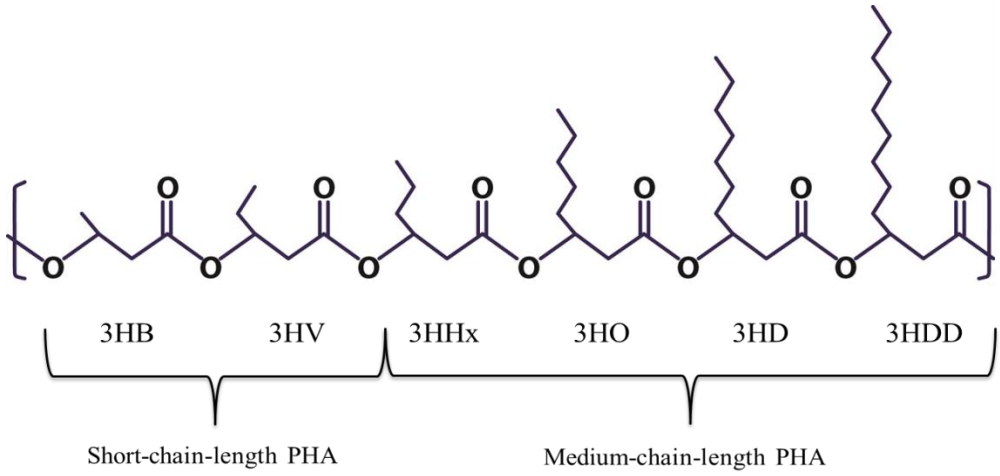


Figure 1.4: PHA copolymer showing the structure of scl and mcl monomers (Chen 2010)

(Scl monomer - 3HB: 3-hydroxybutyrate and 3HV: 3-hydroxyvalerate; mcl monomer - 3HHx: 3-hydroxyhexanoate, 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate and 3HDD: 3-hydroxydodecanoate)

Depending on the monomers in PHAs, they are classified as homopolymers and heteropolymers. The former is formed by polymerization of only one monomer unit eg

PHB whereas the latter is formed by polymerization of two or more different monomeric units eg copolymer of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) P(3HB-co-4HB) (Loo and Sudesh 2007).

1.3 Properties of PHAs

1.3.1 Biological properties

PHAs are carbon and energy storage compounds which usually are accumulated during unbalanced growth condition, i.e. when essential nutrients such as nitrogen or phosphorus are limited in the presence of an excess carbon source. They are high molecular weight compounds and the masses ranges between 20,000 to 30,000 million Daltons. They are hydrophobic, water insoluble granules in the cytoplasm contributing up to 90 % of the cellular dry weight without disturbing the osmotic pressure of the cell (Anderson and Dawes 1990; Chen 2010).

PHAs are lipid-like compounds in which the average number of granules ranges between 8-13 per cell and 0.2-0.5 μm in size depending on the bacterial species. PHA granules are amorphous in nature as crystalline PHA granules do not serve as a source of carbon and energy inside the microorganism. PHA granules are enclosed in a membrane which mainly consists of a phospholipid monolayer and four major granule-associated proteins consisting of PhaC, intracellular PHA depolymerases (PhaZi), phasins (PhaP) and regulator protein of the phasin expression (PhaR) (Madison and Huisman 1999; Ojumu et al. 2004).

PHA granules can be observed as light refracting granules without staining under phase contrast microscope. The granules can also be stained and viewed under a light microscope using lipophilic dyes such as Sudan black B (Murray et al., 1994). PHA

granules exhibit a strong orange fluorescence with Nile blue A stain (Ostle and Holt 1982). Since microorganisms produce PHA hydrolases and depolymerases, PHAs are degraded into carbon dioxide and water in the aerobic environmental condition. Under anaerobic condition, they are degraded into carbon dioxide and methane (Madison and Huisman 1999).

1.3.2 Thermal properties

The properties of PHA copolymer mainly depend on the type and distribution of comonomer units in the polymer (Noda et al. 2005). The melting temperature (T_m), % crystallinity and glass transition temperature (T_g) are the parameters for the thermal characterization of PHAs. The T_m of scl PHA such as PHB is found to be high at 180°C which is close to the thermal decomposition temperature of the polymer. The T_m of PHB can be reduced by the incorporation of 3HA units (such as 3HV, 3HHx and so on) to its backbone. Initially, scl copolymer of PHBV was produced with reduced T_m by the incorporation of a sufficient amount of 3HV units. However, it was difficult to reduce the T_m (< 150°C) and crystallinity of PHBV due to the isodimorphism phenomenon (Holmes 1985; Noda et al. 2005). Later, it was found that the melt temperature of PHAs can be efficiently lowered by the incorporation of mcl monomer units of at least three carbon atoms or more such as 3HHx, 3HO, 3HD and 3HDD units to the predominant PHB monomer (Noda et al. 2010). This mcl monomer incorporation effectively reduces the T_m of the copolymer as seen in Figure 1.5.

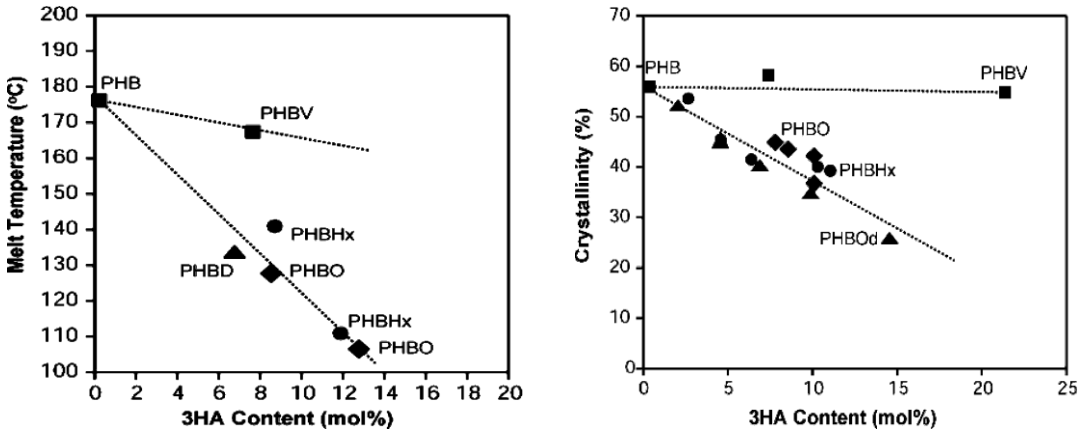


Figure 1.5: Melt temperature and crystallinity of Copolymers (Noda et al. 2005)

Microbially synthesized PHB is 100 % stereospecific and hence highly crystalline, often greater than 50 % (Ojumu et al. 2004). Since crystallinity influences the material stiffness, PHB is a hard and brittle material which limits practical application. As the T_m is lowered by mcl incorporation, crystallinity is also decreased. Therefore with the increase of % mcl monomers containing more than three carbon atoms in PHB, crystallinity of the copolymer decreases to 20-40 % and T_m to 100°C - 150°C to make a material similar to polyethylene. T_g is associated with the stiffness of the material and is inversely proportional to the mol % of the mcl incorporated in the copolymer. Therefore PHB homopolymer has a higher T_g value than the copolymers of PHB (Noda et al. 2010).

1.3.3 Mechanical properties

Scl PHAs (eg PHB) are rigid and highly crystalline and therefore have a stiff and brittle nature. Therefore they show lower ductility and impact strength with high modulus (Sánchez et al. 2003). The scl PHA copolymer (i.e. PHBV) have a decreased stiffness and increased toughness due to the presence of 3HV monomer. However these materials are still rigid which limits their applications.

Mcl PHAs are highly elastomeric and rubbery in nature due to the low degree of crystallinity and lower melting temperature. Therefore they exhibit poor mechanical strength (Hazer and Steinbüchel 2007). The rubbery nature of the plastic is due to the T_g value below room temperature (Shi 2006). Both scl and mcl PHAs possess limitation which hinders their commercial application.

These limitations were overcome by scl-mcl PHA copolymers which have improved ductility and strength due to low crystallinity. For example, Nodax^{TM} copolymers are the family of copolymers which mainly consist of PHB with a small amount of mcl 3HA units comprising carbon units greater than three or more (Noda et al. 2005). This mcl 3HA incorporation into PHB has a pronounced effect, not only by decreasing stiffness and increasing elongation of the material, but also produced more soften material and thus had reduced modulus value.

1.4 *Cupriavidus necator* – model organism of PHA biosynthesis

Cupriavidus necator H16 (formerly called *Hydrogenomonas eutropha, Alcaligenes eutrophus, Ralstonia eutropha* and *Wautersia eutropha*) is a nonpathogenic, Gram-negative bacterium found in soil and freshwater biotopes belonging to the β subclass of the Proteobacteria (Davis et al. 1969; Schlegel et al. 1961; Vandamme and Coenye 2004;

Vaneechoutte et al. 2004; Yabuuchi et al. 1995). It is a lithoautotrophic bacterium which has the ability to use both CO_2 and H_2 in the absence of organic compounds. Apart from the above biological consideration, the main significance of this "knallgas" bacterium is its versatility to accumulate PHB as high as 90 %. The organism is considered as the best PHB producer and the organism used in the commercial production of thermoplastics under the tradename Biopol (Park et al. 2011; Steinbüchel 1992).

The history of research in *C. necator* was commenced in 1960s, when it was first isolated from a spring near Göttingen (Schlegel et al. 1961; Wilde 1962). Studies initiated with the understanding of the physiology of PHA accumulation, the biosynthetic pathway involved and biomass production. In 1970s, isolation of PHB negative mutants was employed for an in-depth understanding of PHB production (Schlegel et al. 1970). In the later 1980s, cloning of three genes involved in PHB synthesis were done independently in three laboratories and further led to the expression of these genes in different microorganisms to confer a functional pathway in them (Reinecke and Steinbüchel 2008). Additional investigation with *C. necator* revealed the production of 3HA other than HB (Steinbüchel and Schlegel 1991; Steinbüchel and Valentin 1995), containing sulfur atoms in the side-chain (Ewering et al. 2002) or even mercaptoalkanoic acids (Lütke-Eversloh et al. 2002a, b; 2001a, b). The genomic sequence of the bacterium has been reported and includes Chromosome 1 (4,052,032 base pairs (bp)), chromosome 2 (2,912,490 bp) and megaplasmid pHG1 (452,156 bp) (Pohlmann et al. 2006; Reinecke and Steinbüchel 2008).

1.5 Metabolic pathway for PHA production

There are different pathways for production of scl and mcl PHAs. Pathway I is used by *C. necator* where PHB is accumulated through three steps: initially β-ketothiolase (*phaA*$_{Cn}$) condenses two acetyl-CoA molecules to form acetoacetyl-CoA which is reduced to R-3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase (*phaB1*$_{Cn}$) and finally, polymerization by PHA synthase (*phaC1*$_{Cn}$) to form PHB as shown in Figure 1.6 (Mifune et al. 2010).

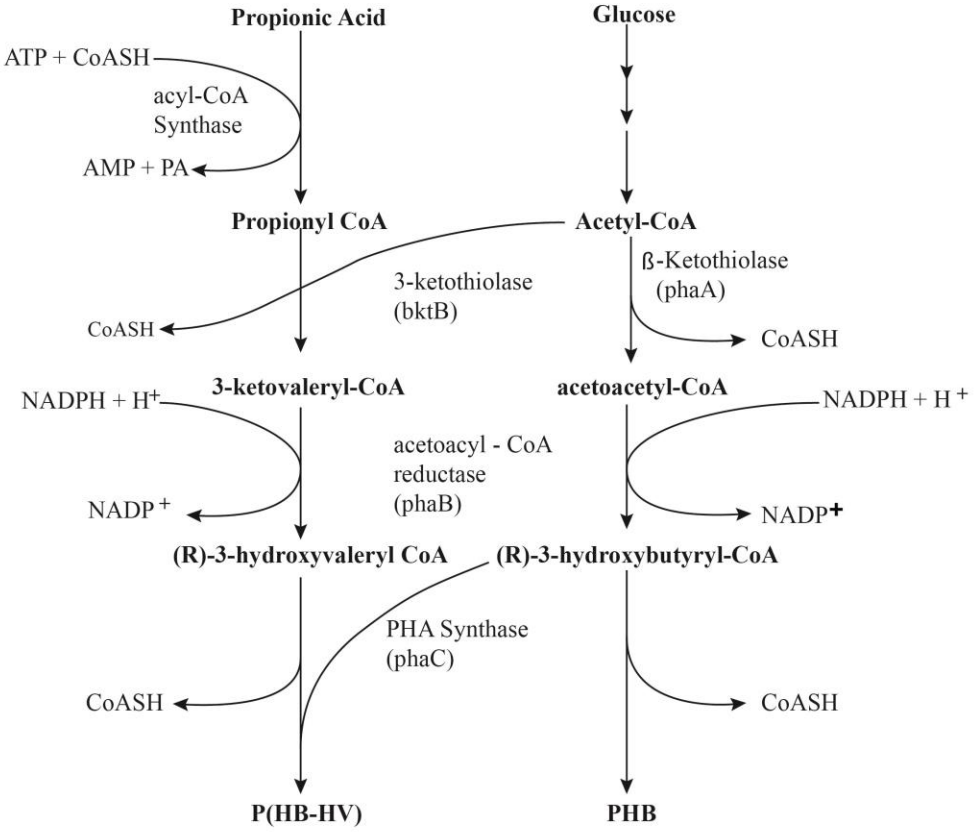


Figure 1.6: The biosynthetic pathway of PHB and PHBV in *C. necator* (Ojumu et al. 2004)

The flux of acetyl-CoA into Tricarboxylic acid (TCA) cycle and PHB biosynthesis pathway highly depends on the growth condition. Under balanced growth, the activity of

β-ketothiolase is inhibited by the free CoA generated and therefore, acetyl-CoA enters the TCA cycle. When non-carbon nutrient limitation is imposed, the NADH/NAD^+ ratio increases, resulting in the inhibition of citrate synthase and isocitrate dehydrogenase. Later, excess acetyl-CoA is channelled into PHB biosynthesis (Anderson and Dawes 1990).

The fatty acid β-oxidation pathway (Pathway II) was deduced in pseudomonads belonging to the rRNA-homology-group I such as *Pseudomonas oleovorans*, *P. putida* and *P. aeruginosa*. They were found to accumulate mcl PHA from various alkanes, alkanols, alkanoates or plant oils. Brigham et al. (2010) reported the existence of two potential fatty acid β-oxidation operons: A0459-A0464 and A1526-A1531 from the genome of *C. necator* H16. Fatty acid β-oxidation is a four step spiral metabolic pathway in which fatty acids are initially activated by acyl-CoA synthetase (*fadD3* – A3288) to form acyl-CoA. In the first step, acyl-CoA is dehydrogenated by acyl-CoA dehydrogenase (*fadE*: A0460, A1530) to form trans-2-enoyl-CoA which is hydrated by 2-enoyl-CoA hydratase (*fadB*: A0464, A1526) to produce (S)-3-hydroxyacyl-CoA which is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase (*fadB*: A0461, A1531) to 3-ketoacyl-CoA which is cleaved by β-ketothiolase (*fadA*: A0462, A1528) to yield a molecule of fatty acyl-CoA that are two carbons less and acetyl-CoA. The fatty acyl-CoA produced has two carbon less can re-enter β-oxidation cycle. Fatty acid β-oxidation is linked to mcl PHA biosynthesis by enoyl-CoA hydratase (*phaJ*) and 3-keto-acyl-CoA reductase (*fabG*) as (R)-3-hydroxyacyl-CoA serves as the substrate for PHA synthase as seen in Figure 1.7 (Fiedler et al. 2002).

Several different pathways are available for the production of mcl monomers from non-related carbon sources such as glucose, fructose and sucrose (Philip et al. 2007). Fatty acid *de novo* biosynthesis is the one such pathway (Pathway III) which is involved in the generation of fatty acids by building up two carbons per cycle through acyl carrier protein (ACP) linked intermediates. *Pseudomonas sp.,* like *P. aeruginossa, P. aureofaciens, P. citronellolis* and *P. putida* are reported to have this pathway. The gene acyl-CoA-ACP transferase encoding *phaG* is an enzyme linking the fatty acid biosynthesis pathway to the mcl PHA biosynthesis pathway. This enzyme transfers the hydroxyacyl moiety from (R)-3-hydroxy-acyl carrier protein to coenzyme A, thus forming (R)-3-hydroxyacyl-CoA, which acts as the substrate for the PHA synthase enzyme (Sudesh et al. 2000).

In the entire above biosynthetic pathway, PHA synthase plays a vital role in polymerizing the monomer to produce polymer. It is a key enzyme determining the type of PHA synthesized by the microorganism. About 59 different PHA synthase have been obtained from 45 different bacteria. Depending on the subunit composition and specificity of the enzyme, four major classes of PHA synthase exist.

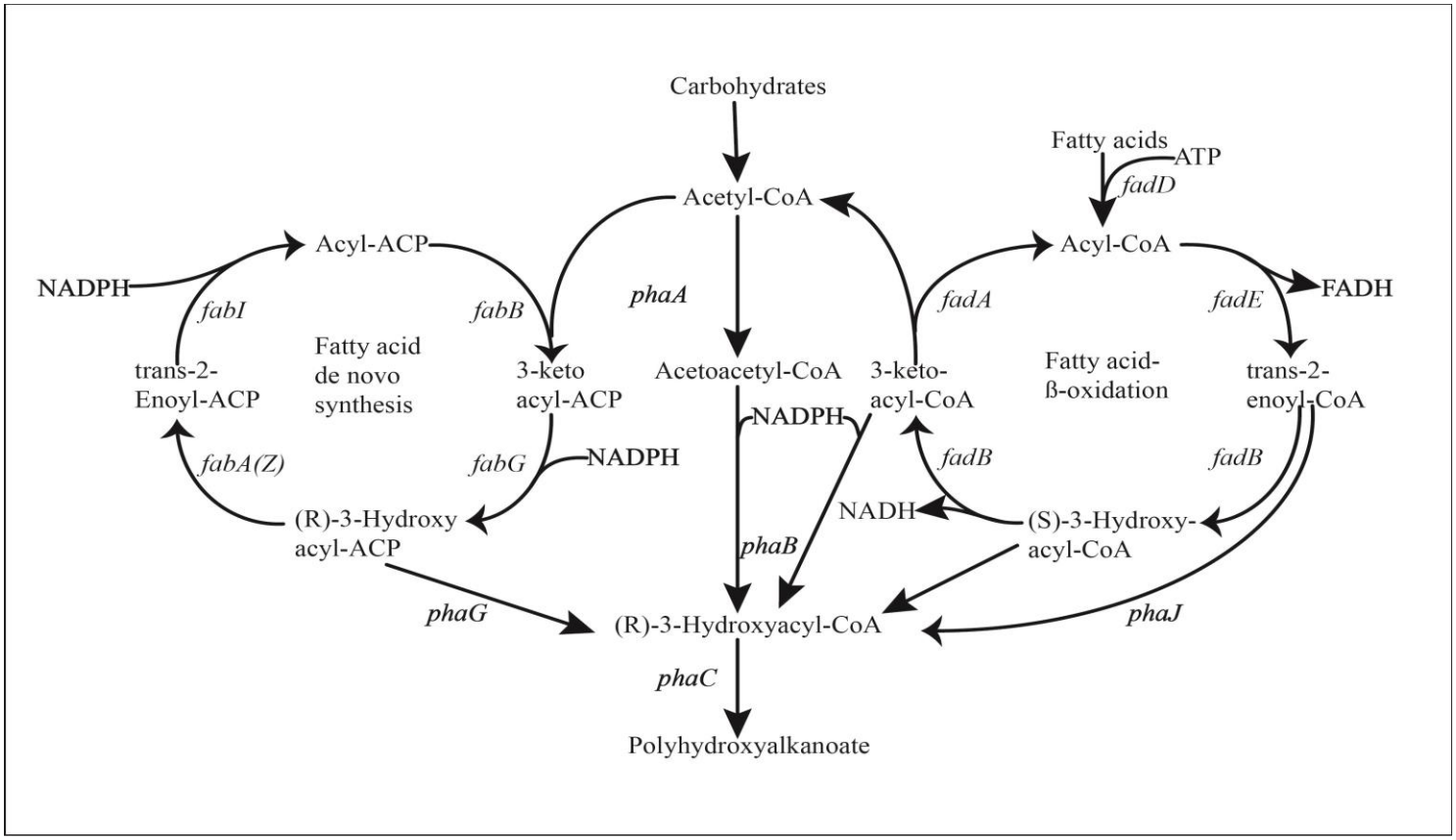


Figure 1.7: PHA biosynthesis in the context of microbial metabolism. Modified from Aldor and Keasling (2003)

Abbreviations: PhaA, 3-ketothiolase; PhaB, (R)-3-ketoacyl-CoA reductase (for PHB biosynthesis, this enzyme is acetoacetyl-CoA reductase); PhaC, PHA synthase or polymerase; PhaG, (R)-3-hydroxyacyl ACP:CoA transacylase; PhaJ, (R)-specific enoyl-CoA hydratase. PhaC is specific for enantiomeric monomers in the (R) configuration.

Table 1.1: Classes of PHA synthase (Sudesh 2013)

Class	Subunits and average size	Representative species	Substrate
I	PhaC ~ 60-73 kDa	*Cupriavidus necator*, *Sinorhizobium melioti*, *Burkholderia sp*	$3HA_{scl}$-CoA (~C3-C5) $4HA_{scl}$-CoA, $5HA_{scl}$-CoA
II	PhaC ~ 60-65 kDa	*Pseudomonas aeruginosa*, *Psedomonas putida*	$3HA_{mcl}$-CoA (~≥C5)
III	PhaC-PhaE*	*Allochormatium vinosum*, *Thiocapsum pfennigii*, *Synechocystis sp. PCC6803*	$3HA_{scl}$-CoA ($3HA_{mcl}$-CoA (~C6-C8), $4HA_{scl}$-CoA, $5HA_{scl}$-CoA)
IV	PhaC-PhaR ~ 40 kDa, ~22 kDa	*Bacillus megaterium*, *Bacillus sp. INT005*	$3HA_{scl}$-CoA

* - Size not reported

Class I PHA synthase comprises only one subunit PhaC with the molecular mass of 61 KDa and utilize CoA thioesters of C3-C5 carbon atoms (eg *C. necator*) (Qi and Rehm 2001). Class II PHA synthase, similar to class I, comprise only one subunit with 73 KDa and utilize CoA thioesters of C6-C14 carbon atoms (eg *P. aeruginosa*) (Amara and Rehm 2003; Peoples and Sinskey 1989; Schubert et al. 1988; Slater et al. 1992; Slater et al. 1988). Class III PHA synthase (eg *Allochromatium vinosum*) comprises two subunits; a PhaC subunit (40 KDa) and a PhaE subunit (40 KDa) and prefers CoA thioesters of C3-C5 carbon atoms (Liebergesell et al. 1992; Yuan et al. 2001). Class IV PHA synthase (eg *Bacillus megaterium*) resembles class III PHA synthase where PhaR (20 KDa) is present instead of PhaE subunit (McCool and Cannon 2001). An exception is the PHA synthase from *Thiocapsa pfennigii* which has broad substrate specificity for both CoA thioesters of C3-C5 and C6-C14 carbon atoms (Table 1.1) (Fukui and Doi 1997; Liebergesell et al. 2000; Matsusaki et al. 1998).

1.6 Nutrient requirements and limitations for PHA accumulation

There was considerable interest to establish the physiology and metabolism of *C. necator*. Repaske (1962, 1966) reported the nutritional requirement and characteristics for its growth. Repaske and Repaske (1976) established the quantitative nutritional requirement for the continuous autotrophic growth of *C. necator* and the minimal saturating concentration for Mg^{2+}, $S0_4^{2-}$, $P0_4^{3-}$, Fe^{3+} and Na^{2+} were 10^{-4} M, 8 x 10^{-5} M, 5 x 10^{-4} to 6 x 10^{-4} M, 10^{-5} M and 10^{-7} to 2 x 10^{-7} M, respectively. A deficiency of iron results in culture foaming and later by the appearance of a yellow-green pigment in the culture. In 1970s and in 1980s, efforts were made to study their characteristic growth, cell composition, kinetics and modeling of PHB production (Doi et al. 1988; Heinzle and Lafferty 1980; Morinaga et al. 1978; Mulchandani et al. 1989). In parallel, production processes have also been developed for commercial production by Imperial Chemical Industries in 200,000-liter stirred fermentation vessels (Madison and Huisman 1999).

PHAs are synthesized in microorganisms as a result of a one or more limitations on growth such as oxygen ‘O’, nitrogen ‘N’, phosphorus ‘P’, sulphur ‘S’, magnesium ‘Mg’, or potassium ‘K’. Magnesium is vital for cell growth and function, maintaining the integrity of the ribosomes, to control permeability of cell membranes and as a cofactor in many enzymatic reactions. Inorganic phosphate is an essential cell component, as it is found in nucleic acids, phospholipids, proteins and coenzymes (Asenjo et al. 1995). Another way to induce nutrient limitation is via nitrogen by adjusting the C/N ratio in the medium composition. The C/N ratio plays a vital role in regulating and promoting PHA synthesis. In principle, active growth and metabolism is exhibited at lower C/N ratios whereas high C/N ratios results in N-limitation which promotes higher PHAs accumulation (El-Sayed et al. 2009; Yu et al. 1998).

1.7 PHA production

1.7.1 Wildtype *C. necator*

The choice of microorganism for the industrial production of PHA has to meet certain criteria such as the cell's ability to grow in an inexpensive substrate, achieve a high yield, have a high polymer synthesis rate and the maximum % of PHA accumulation (Lee 1996). Diverse species of bacteria are known to accumulate PHB but only a few of them are employed in a production process and included *Ralstonia eutropha* (Ryu et al. 1997), *Alcaligenes latus* (Wang and Lee 1997), *Azotobacter* v*inelandii* (Chen and Page 1997; Page and Cornish 1993), several strains of methylotrophs (Kim et al. 1996) and recombinant *Escherichia coli* (Wang and Lee 1997). Bacteria that are employed in PHA production are classified into two groups. The first group requires nutrient limitation for the production of PHAs eg *C. necator*, *Protomonas estorquens* and *P. oleovorans*. The second group produces PHA during growth eg *Alcaligenes latus*, and a mutant strain of *Azotobacter vinelandii* (Lee 1996). These bacteria are capable of growing to high cell density cultures (HCDC) with a high amount of PHB accumulation. Efforts have been made to develop a suitable fermentation process depending on the microorganism and growth conditions to improve PHA productivity (Lee et al. 1999b).

C. necator has been widely studied for its ability to produce PHB, up to 80-90 %, from simple carbon sources such as glucose or fructose employing N or P limitation (Repaske and Mayer 1976). Khanna and Srivastava (2006) showed that the batch kinetics of *C. necator* featured 19.7 g/l biomass and 10.89 g/l of PHB accumulated in 60 h in statistically optimized culture conditions.

The primary objective of the fermentation process is cost effective production with high productivity and yield. In general, a fed batch process is superior to batch, as the change in carbon source concentration can affect the productivity and yield of the process. Also development of a suitable strategy is important in a fed batch process as both over or under feeding is detrimental to the survival of the organism and affects product formation (Lee et al. 1999a).

Khanna and Srivastava (2006) conducted a fed batch cultivation process by alternate feeding of nitrogen (urea) and carbon (fructose) substrates where 18.46 g/l of PHB was accumulated in 40 h with the overall productivity of 0.46 g/l·h. *C. necator* was cultivated in glucose and propionic acid in a two stage cultivation process to accumulate PHBV by imposing N-limitation. The mol % of 3HV varied from 0-50 % with the ratio of glucose to propionic acid (Doi et al. 1987).

Numerous strategies have been developed for intermittent or continuous feeding of substrate into the fermenter based on monitoring the dissolved oxygen (DO), pH or a carbon source concentration as a control parameter. DO-stat and pH stat are simple, yet the substrate concentration cannot be maintained at a desired level which reduces the ease of the fermentation process (Ryu et al. 1999). Kim et al. (1994a) obtained a high cell concentration of 164 g/l with a PHB concentration of 121 g/l in *A. eutrophus* NCIMB 11599 using an on-line glucose analyzer. Such studies employed a complex and expensive online analysis. At the end, the concentration of glucose was fairly maintained between 5-30 g/l (Ryu et al. 1999), in which the optimal glucose concentration for the growth of this organism was found to be 10 to 20 g/l (Kim et al. 1994a).

The capability of *C. necator* strain H16 to metabolize sugars is restricted to fructose (Pohlmann et al. 2006) and other carbon sources that include volatile fatty acids such as acetate, propionate, lactate, butyrate, valerate and plant oils (Madison and Huisman 1999). The product yield mainly depends on the type of carbon and energy source utilized. The yields of PHA from glucose or vegetable oil have been reported to be 0.38-0.46 g-PHB/g-glucose (Ryu et al. 1997) and 0.6-0.8 g-PHA/g-oil respectively (Ng et al. 2010).

High yield production of PHAs was done with *C. necator* using soybean oil as carbon source where a high cell density of 118-126 g/l was obtained with 72-76 % w/w PHB (Kahar et al. 2004). Ng et al. (2010) utilized jatropha oil to produce PHB from the wildtype *C. necator* and accumulated 87 % w/w PHB.

C. necator possesses class I PHA synthase and preferentially utilizes CoA thioesters of C3-C5 carbon atoms to produce scl-PHAs. However, Green et al. (2002) showed that *C. necator* is capable of incorporating mcl PHAs such as 3HHx and 3HO, when sodium carbonate as the carbon source was used with a β-oxidation inhibitor such as acrylic acid. López-Cuellar et al. (2011) were able to synthesize mcl PHAs from fructose and canola oil to accumulate 95 % PHB with 5 % of 3HB, 3HV, 3HO, and 3HDD.

1.7.2 Recombinant *C. necator*

Although wildtype *C. necator* has been widely studied for PHA production, recombinant strains of *C. necator* has been developed to improve the productivity and to control the monomer composition of the copolymer (Jung et al. 2010). Production of PHB was studied in transformant *C. necator* harboring *phbCAB* (AER3), *phbAB* (AER4) and *phbC* (AER5) genes. Among them, AER5 showed the efficient cell growth and PHB accumulation, accordingly it was concluded that PHB synthase is the most critical enzyme for PHB biosynthesis. Subsequently, Park et al. (1997) studied the characteristics of cell growth and PHB synthesis by transformant *C. necator*. Batch cultivation of AER5, at high C/N ratio produced a higher amount of PHB. In fed batch cultivation, the fermentation time was reduced and the cell mass, PHB concentration remained high compared to the wildtype.

A recombinant strain of *C. necator* PHB$^-$4 (a PHA-negative mutant), harboring a PHA synthase gene from *Aeromonas caviae*, grown on plant oils such as olive oil, corn oil, palm oil and oleic acid was able to accumulate a random copolyester of 3HB and up to 4-5 mol % 3HHx (Fukui and Doi 1998). Studies by Kahar et al. (2004) of a recombinant strain of *C. necator* PHB$^-$4/pJRDEE32d13 (a PHA-negative mutant harboring *A. caviae* PHA synthase gene, $phaC_{Ac}$) grown on soybean oil produced a copolymer of PHB with 5 mol % 3HHx with a dry cell weight of 128-138 g/l and a high PHA content of 71-74 % (w/w). Recombinant *C. necator* PHB$^-$4/pJRDEE32d13 was able to synthesize poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) P(3HB-co-3HHx) with 3 mol % of HHx using jatropha oil as carbon source (Ng et al. 2010).

Numerous studies are available with recombinant *C. necator* which mainly focused on the manipulation of critical enzymes such as PHB synthase as described

above. Heterologous expression of enzymes *phaG*$_{Ps}$ encoding 3-hydroxyacyl acyl carrier protein coenzyme A transacylase and the *phaC1*$_{Ps}$ genes encoding PHA synthase from *Pseudomonas sp*. 61-3 was made in a negative mutant of *C. necator* PHB^{-}4. PHB was accumulated to 96-97 mol % with ~3 mol % C6-C12 mcl units from sugars (Matsumoto et al. 2001).

Recombinant *C. necator* capable of producing P(3HB-co-3HHx) from fructose was constructed by the introduction of genes for crotonoyl CoA reductase from *Streptomyces cinnamonensis* (*ccr*$_{Sc}$) and PHA synthase, (R)-specific enoyl CoA hydratase from *A. caviae* (*phaC-J*$_{Ac}$) and was able to accumulate up to 1.5 mol % 3HHx from fructose (Fukui et al. 2002).

Currently, Riedel et al. (2012) used an engineered strain of *C. necator* Re2058/pCB113 harboring *PhaC2*$_{Ra}$ from *Rhodococcus aetherivorans* I24 and *PhaJ1*$_{Pa}$ from *P. aeruginosa* in a high cell density cultivation using palm oil to produce a different copolymer, P(3HB-co-3HHx).

1.8 Applications of polyhydroxyalkanoates

PHAs has a wide range of properties and valuable application. In the packaging industry, they have been used to make bags, sizing agent, containers and coat paper (Bourbonnais and Marchessault 2010). Other applications in products used daily include razors, food utensils, diapers, feminine hygiene products, shampoo bottles and cups (Khanna and Srivastava 2005). PHA is widely used in industrial application as renewable biofuels (Zhang et al. 2009). Due to the limited applications of PHB, its copolymer has wider application in the packaging industry (Gumel et al. 2012). The copolymers of PHB such as PHBV, P(3HB-co-3HHx) and other PHAs are used in medical applications as bone plates, osteosynthetic materials, surgical sutures and in the slow release of drugs and hormones (Philip et al. 2007). PHA was found to be useful in pathogen control for example PHB inhibits the gut microflora of the fresh water prawn *Macrobrachium rosenbergii* (Nhan et al. 2010). In agriculture, it can be used as controlled release agents for herbicides (Grillo et al. 2011). Some of the examples of commercially available PHAs are BIOPOL, Nodax^{TM} and DegraPol.

2 Justification

The first PHA (i.e. PHB) was discovered and characterized by Lemoigne in the 1920s and found to be useful as a bioplastic by Baptiste in the 1950s. Its high melting point (near its degradation temperature) and low ductility prevented commercialization until methods were developed to feed suitable substrates to produce lower melting, less crystalline co-polymers. This was possible since the PHB synthase can accept slightly larger carbon substrates (C5 in addition to C4) and led to the commercialization of PHBV by Imperial Chemical Industries. In the early 1980s, *P. oleovorans* (now *putida*) and related organisms were discovered to produce another kind of PHA. This lower melting, elastomeric PHA could be accumulated from mcl carboxylic acids due to a different PHA synthase (Type II). It became doctrine that the original PHA synthase (Type I) could only accumulate C4 and C5 substrates (Haywood et al. 1989) until it was shown that the Type I synthases of *Rhodospirillum rubrum* (Brandl et al. 1989), *Thiocapsa pfennigii* (Libergesell at al. 1993) and *Aeromonas caviae* (Shimamura et al. 1994) could also polymerize significant amounts of C6 3HA. This knowledge became of commercial importance when Proctor and Gamble patented scl-mcl PHAs under the trade name of NODAX (Noda 1996). It was believed that such materials could only be produced using special PHA synthases that could accumulate both scl and mcl substrates but it was eventually demonstrated that even the Type I synthase of *C. necator* could accept C6 and possibly larger substrates albeit with less affinity than some other synthases (Dennis et al. 1998). It was subsequently shown that, by manipulating the culture conditions (in this case inhibiting beta-oxidation), substantial amounts of 3HHx could be incorporated into

so-called scl-PHA using the *R. eutropha* synthase with sodium octanoate (Green et al. 2002).

More recently, it was demonstrated that less expensive substrates, plant oils, could be used by a bacterium with a Type I synthase to produce PHA with much lower crystallinity and melting point than was previously considered possible. Unfortunately, not enough polymer has been produced to determine the mechanical properties of these materials. López-Cuellar et al. (2011) has so far achieved the highest biomass production in a three stage process with most of the polymer accumulating in the third stage under N-limitation with canola oil as the carbon substrate. The copolymer was primarily 3HB and small amounts of 3HV, 3HO and 3HDD. However, only a maximum of 19 g/l of PHA was produced. There are no other reports on the bench scale production of scl-mcl PHA by *C. necator* H16. In order to produce sufficient material for testing and to develop applications, the polymer must be produced to a higher level. While this bacterium has been successfully grown to high cell density using soybean oil as sole source of carbon, no mcl PHA was detected (Kahar et al. 2004). In other studies, glucose was the sole substrate used for the production of the PHB homopolymer (Kim et al. 1994; Ryu et al. 1999). Although carbohydrate substrates like glucose are less expensive than vegetable oils like soya bean oil, mcl PHAs have not been shown to be produced by wildtype *C. necator* from carbohydrates alone, and only López-Cuellar et al. (2011) has shown that scl-mcl PHA can be produced by wildtype *C. necator* using fructose for growth and canola oil for polymer accumulation.

In this study, the primary focus was on the co-expression of genes (for improved oil metabolism) to enhance the incorporation of canola derived mcl monomer into scl-mcl

PHAs copolymer produced by transformant *C. necator*. Firstly, acyl-CoA dehydrogenase *($fadE_{Ec}$* from *E. coli)* is known as the rate-limiting enzyme for the β-oxidation pathway and it is necessary for the conversion of fatty acids to enoyl-CoA. Secondly, enoyl-CoA *($phaJ1_{Pp}$* from *P. putida)* is one of the key intermediates linking fatty acid β-oxidation with the PHA synthesis pathway.

Finally, the main target of this work was to increase the density of wildtype and transformant *Cupriavidus necator* biomass grown on fructose in order to produce sufficient copolymer of scl and mcl PHA from canola oil. This PHA copolymer produced was used for the thermal and mechanical characterization.

3 Hypothesis

1. Co-expression of acyl-CoA dehydrogenase (*fadE*$_{Ec}$ from *E. coli)* and enoyl-CoA hydratase (*phaJ1*$_{Pp}$ from *P. putida)* in transformant *C. necator*, under an *araC-P*$_{BAD}$ promoter system, will enhance the proportion of mcl monomer into the scl-mcl PHA copolymer than the wildtype.

2. A three stage feeding strategy for higher biomass production in wildtype and transformant *C. necator* will produce sufficient material for thermomechanical testing of the PHA.

3. Thermomechanical characterization of the scl-mcl PHA copolymer produced by the transformant will show improved material properties than the wildtype.

4 Objectives

4.1 Main objective

Improved incorporation of canola derived mcl monomers from the transformant strain of *C. necator*. Evaluation on the thermomechanical properties of scl-mcl PHA copolymer produced by the high cell density cultivation of wildtype and transformant *C. necator*.

4.2 Secondary objectives

1. To construct a transformant *C. necator* with acyl-CoA dehydrogenase (*fadE*$_{Ec}$ from *E. coli)* and enoyl-CoA hydratase (*phaJ1*$_{Pp}$ from *P. putida)* for enhancing the mcl monomer proportion into the scl-mcl PHA copolymer.
2. To establish a three stage feeding strategy from fructose and canola oil by the wildtype and transformant to produce sufficient copolymer material for the thermomechanical testing of the PHA.
3. To compare the material properties and its effect on the incorporation of mcl monomers into PHB produced by the wildtype and transformant *C. necator*.

5 Materials and Methods

5.1 Cloning and expression of *fadE*$_{Ec}$ and *phaJ1*$_{Pp}$ in *C. necator*

5.1.1 Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 5.1. *C. necator* and *E. coli* strains were grown in Luria-Bertani (LB) broth (g/l) (tryptone 10; yeast extract 5; NaCl 5) at 30 and 37°C respectively. Ampicillin (100 µg/ml), kanamycin (50 µg/ml) or chloramphenicol (25 µg/ml) was added as required. All the enzymes such as restriction endonucleases, Klenow fragments and T4 DNA ligase were purchased from New England Biolabs.

5.1.2 Cryopreservation of bacteria

The bacterial strains were cryopreserved in glycerol as described by Malik (1991). Briefly, bacterial cells were grown in LB broth for 16-18 hrs at 37°C. 500 µl of sterile ice cold 30 % glycerol was added to 500 µl of bacterial cells in cryovial and mixed gently. The vials were then stored at -80°C.

Table 5.1: Bacterial strains and plasmids

Strains or Plasmids	Characteristic feature	Source or reference
Bacteria		
E. coli DH5α	Competent cells	Lab stock
C. necator H16	American Type Culture Collection (ATCC – 17699)	Manassas, VA
Genes		
$fadE_{Ec}$	*E. coli str. K-12*	
	GS45640-1 pBSK gene 1	Synthesized from Epoch
	GenBank Accession NC_000913	Biolabs Inc, Missouri
$phaJ1_{Pp}$	*P. putida KT2440*	City, TX, USA
	GS45640-1 pBSK gene 2	
	GenBank Accession NC_002947	
Plasmid		
pBTB-3	Origin of replication pBBR1, promoter	Lynch and Gill (2006)
	P_{BAD}, promoter CAT, Cm^R, t*onB* ter	
pK18	Origin of replication ColE1, Km^R	Pridmore (1987)
pMPJAS01	Fusion of plasmids pBTB-3 and pK18	Present work
pMPJAS02	pBSK gene 1 harboring genes $fadE_{Ec}$ & $phaJ1_{Pp}$	Present work
pMPJAS03	Plasmid containing pMPJAS01 and $fadE_{Ec}$ - $phaJ1_{Pp}$	Present work
	downstream of $araC$-P_{BAD} promoter	

5.1.3 DNA manipulation

5.1.3.1 Preparation of competent cells

A freshly grown culture (0.2 ml) was transferred to 5 ml of SOB medium g/l (tryptone 20; yeast extract 5; NaCl 0.5) containing 50 µl of solution III (10 mM of 1 M $MgSO_4$ and 1 M of $MgCl_2$) and the cells were incubated overnight at 37°C. 0.5 ml of overnight culture was inoculated with 50 ml of SOB complemented with 0.5 ml of solution III. The OD measurements were taken at 595 nm after 2 ½-3 hrs, till it reached 0.5. The cells were centrifuged at 3,000 rpm for 15 minutes at 4°C then resuspended in 16 ml of TFB 1 (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % glycerol. pH adjusted to 5.8 with 0.2 M acetic acid, sterilized and stored at 4°C) solution and incubated for 15 minutes. Again, they were centrifuged at 3,000 rpm for 15 minutes at 4°C and resuspended in 4 ml of TFB 2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM rubidium chloride, 15 % glycerol. The pH was adjusted to 6.5 with KOH. Filtered, sterilized and stored at 4°C) solution and incubated for 15 minutes. The cells were distributed in aliquots of 200 µl in the Eppendorf tubes and stored at -80°C (Hanahan 1983).

5.1.3.2 *E. coli* DH5α transformation

An aliquot of the competent cells were taken and mixed with 2 µl of the plasmid DNA and placed in ice bath for 30-45 minutes. Then the mixture is placed in water bath for 90 sec at 42°C. Later it was placed in the ice bath for 45 minutes. 800 µl of SOB medium was added and incubated in a shaken for 1 h 20 min. Then the cells were centrifuged and resuspended in 100-200 µl of final volume of the media and plated with appropriate

antibiotics. 100 µl of culture was inoculated in case of control DNA plates containing appropriate antibiotics.

5.1.3.3 Plasmid DNA extraction

Mini-preparation of plasmid DNA was obtained by alkaline lysis method (Sambrook and Russell, 2001). A single bacterial colony was transferred into 2 ml of LB medium containing appropriate antibiotic in a loosely capped 15 ml tube. The culture was incubated overnight at 37°C with vigorous shaking. 1.5 ml of the culture was poured into a microfuge tube. The cells were centrifuged at 12,000×*g* for 30 sec at 4°C in a microfuge. The reminder of the culture was stored at 4°C. The culture medium is removed by aspiration, leaving the pellet as dry as possible. This extraction protocol is a modification of the methods of Bimboim and Doly (1979) and Ish-Horowicz and Burke (1981). The bacterial pellet was resuspended in 100 µl of ice cold solution I (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0) which was dispersed completely by vigorous vortexing. 200 µl of solution II (0.2 N NaoH, 1 % SDS) was added and the contents were mixed by inverting the tubes rapidly 4-5 times. The tube was then incubated on ice. 150 µl of ice cold solution III (5 M potassium acetate - 60 ml, glacial acetic acid - 11.5 ml, H_20 - 28.5 ml) was added. The tubes were vortexed gently in an inverted position to disperse the solution III through the viscous bacterial lysate. The tube was stored on ice for 3-5 minutes. The tubes were centrifuged at 12,000×*g* for 5 min at 4°C in a microfuge. The supernatant were transferred to a fresh Eppendorf tube. An equal volume of phenol: chloroform (1:1, 50 µl) was added. The resulting mixture was vortexed well and centrifuged at 12,000×*g* for 2 min at 4°C in a microfuge. The supernatant was transferred to a fresh Eppendorf tube and the double stranded DNA was

precipitated with two volumes of ethanol at room temperature. Then the mixture was allowed to stand for 2 min at room temperature. The tubes were centrifuged at 12,000×*g* for 5 min at 4°C in a microfuge. The supernatant was removed by gentle aspiration and the tubes were placed in an inverted position to drain away the solvents. Again, the pellet of DNA was rinsed with 1 ml of 70 % ethanol at 4°C. The supernatant was removed as mentioned earlier and the DNA pellet was dried in air for 10 minutes. The DNA was resuspended in 30-50 µl of distilled water and stored at -20°C for further analysis.

5.1.3.4 Restriction digestion and gel electrophoresis

DNA of 200-1000 ng was used with 1:10 dilution of the 10X buffer with 1 µl of the respective enzyme. The restriction digestion were carried out for 2 h at 37°C and analyzed by gel electrophoresis.

Plasmid DNA was analyzed using agarose gel electrophoresis. The electrophoresed gel was pictured using UV Transilluminator (Hoefer MacroVue, Uvis-20) and were further analyzed by computer.

5.1.3.5 Purification of plasmid DNA

5.1.3.5.1 Phenol: Chloroform

Plasmid DNA of 20-50 µl was made up to 100 µl with distilled water. 50 µl of Tris saturated phenol and 50 µl of chloroform were added and mixed well. The mixture is then centrifuged and the supernatant was transferred to a fresh tube. To this solution, either 20 % of 10 M ammonium acetate or 10 % of 5 M potassium acetate was added and mixed well. Then 2.5 volume of 100 % ethanol was added, mixed well and incubated overnight at -20°C or for 2-3 hrs at -80°C. The tube was centrifuged at 12,000×*g* for 15

min at 4°C in a microfuge and the washing was repeated again with 70 % ethanol. The supernatant was removed and the pellet was air dried.

5.1.3.5.2 Purification of DNA from gel

The sliced DNA from the gel was placed in a squeeze and freeze column (Freeze 'N Squeeze DNA extraction spin column, Biorad). The tube was incubated overnight at -20°C or for 2 - 3 hrs at -80°C. Then the column was centrifuged 12,000×*g* for 3 minutes. To this solution, 20 % of 10 M ammonium acetate or 10 % of 5 M potassium acetate was added and mixed well. Again, 2.5 volume of 100 % ethanol was added, mixed well and incubated overnight at -20°C or for 2-3 hrs at -80°C. The tube was centrifuged at 12,000×*g* for 15 min at 4°C in a microfuge and the washing was repeated again with 70 % ethanol. The supernatant was removed and the pellet was air dried.

5.1.4 Sequence synthesis of $fadE_{Ec}$ and $phaJ1_{Pp}$

The sequence of the gene $fadE_{Ec}$ and $phaJ1_{Pp}$ were obtained from the NCBI database (Table 1). The amino acid with high frequency was tabulated for *C. necator* from the Codon Usage Database. The gene sequences obtained were converted into amino acid sequence using *In silico* simulation (http://insilico.ehu.es/). The amino acid sequence comparison were made using *ClustalW* program to obtain 100 % similarity. The genes $fadE_{Ec}$ and $phaJ1_{Pp}$ were synthesized and their codon optimized for *C. necator* expression (Epoch Biolabs, Missouri City, TX, USA).

5.1.5 Construction of *C. necator* pMPJAS03

All DNA manipulations were performed following standard procedures (Sambrook and Russell, 2001). *E. coli* DH5α was used in all transformation procedures. Recombinant plasmids for the functional expression of $fadE_{Ec}$ and $phaJ1_{Pp}$ in wildtype *C. necator* were

constructed using the broad host range plasmid pBTB-3 and the kanamycin resistance plasmid pK18. Both the plasmids pBTB-3 and pK18 were digested respectively with HindIII and fused together to form pMPJAS01. The plasmid GS45640-1 pBSK gene 1 (*fadE*$_{Ec}$) was linearized with HindIII and blunt ended with Klenow. The plasmid GS45640-1 pBSK gene 2 harbouring *phaJ1*$_{Pp}$ was fragmented by digesting with EcoRV – XhoI and blunt ended with Klenow. Both the linearized plasmid and the gene fragment were ligated to form pMPJAS02. Next, pMPJAS01 was digested with SmaI and linearized. The 3.0 kb *fadE*$_{Ec}$*-phaJ1*$_{Pp}$ DNA fragment from pMPJAS02 was AccI and HindIII digested, blunt ended with Klenow and inserted into pMPJAS01 to form pMPJAS03 (Figure 5.1). The resultant plasmid pMPJAS03 was introduced into *C. necator* by electroporation.

5.1.6 Electrocompetent cells preparation and transformation in *C. necator* pMPJAS03

Electrocompetent cells were prepared according to Sambrook and Russell (2001). In brief, a seed culture of *C. necator* was prepared in 25 ml of LB with an overnight incubation at 30ºC and 200 rpm. 5 ml of this culture was inoculated into 500 ml LB in a 2 l Erlenmeyer flask. The culture was grown to an O.D. (600 nm) of 0.6 - 0.7. The cells were concentrated by centrifugation and washed with an equal volume of ice cold sterilized MQ distilled water three times to remove residual medium. The culture was resuspended in 3 ml of 10 % glycerol and stored as 100 µl in Eppendorf tubes at -80ºC until required.

After 5 min incubation on ice of a cell suspension (100 µl) mixed with 1-5 µg of DNA in an electroporation cuvette (0.1 cm gap width), electroporation was performed at

1.8 kV, 25 μF and 4.0 ms settings in a Gene Pulser II electroporator equipped with a Pulse Controller Plus module (Bio-Rad Laboratories, Hercules, CA).

5.1.7 Isolation of *C. necator* pMPJAS03 plasmid DNA

A rapid alkaline extraction procedure was used for screening recombinant plasmid DNA (Bimboim and Doly 1979) as mentioned earlier.

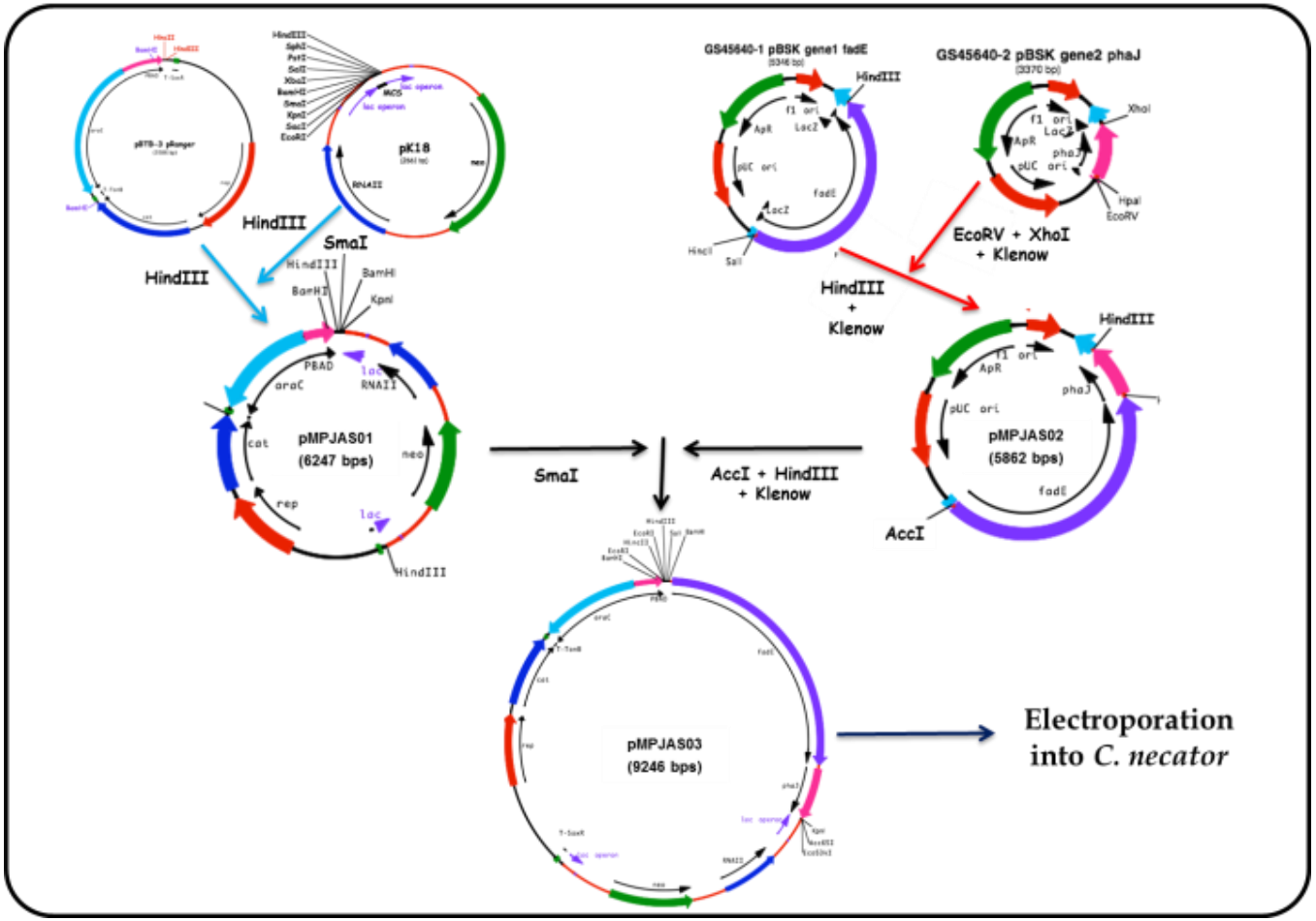


Figure 5.1: Construction of recombinant plasmids: pMPJAS01 (harbouring broad host range plasmid pBTB-3 and the kanamycin resistance plasmid pK18), pMPJAS02 (harbouring genes *fadE*$_{Ec}$ and *phaJ1*$_{Pp}$) and plasmid pMPJAS03 (containing the *fadE*$_{Ec}$-*phaJ1*$_{Pp}$ DNA fragment under *araC*-*P*$_{BAD}$ promoter system)

5.2 High cell density cultivation

5.2.1 Inoculum and growth media

Wildtype *C. necator* H16 (ATCC 17699, obtained from American Type Culture Collection, Manassas, VA) was maintained on nutrient agar plates at 4°C. A 10 % (v/v) inoculum for the bioreactor was prepared in a medium containing per liter: 10 g fructose, 3.70 g $(NH_4)_2SO_4$, 0.40 g $MgSO_4$, 6.36 g $Na_2HPO_4 \cdot 7H_2O$, 2.70 g KH_2PO_4 and 1.00 g nutrient broth. The initial culture medium in the bioreactor contained per liter: 3 g fructose, 4.70 g $(NH_4)_2SO_4$, 0.40 g $MgSO_4$, 7.7 g $Na_2HPO_4 \cdot 7H_2O$, 3.26 g KH_2PO_4 and 10 ml trace element solution. Each liter of trace element solution contained 10 g $FeSO_4 \cdot 7H_2O$, 3 g $CaCl_2 \cdot 2H_2O$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.15 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$ and 1.00 g $CuSO_4 \cdot 5H_2O$. The feed medium for the fed-batch stage contained per liter: 500 g fructose, 12 g $MgSO_4$ and 120 ml trace element solution. Canola oil was used as substrate for the PHA accumulation phase. During growth, 28 % (w/v) NH_4OH was used to control the pH and was replaced by 2 M KOH during the N-limited accumulation stage.

5.2.2 Fermentation studies

All fermentations were performed in a 5 l Minifors bioreactor (Infors HT, Bottmingen, Switzerland) at 30 ± 1°C with a 3 l initial working volume. LabVIEW 6.1 (National Instruments, Austin, Texas) was used to monitor and/or control parameters such as dissolved oxygen (DO), outlet gas CO_2, carbon source feeding and pH. pH (Easyferm Plus K8200, Hamilton Company, USA) was maintained at 7 ± 0.05 by the addition of NH_4OH or KOH. DO (Mettler Toledo, Hamilton Company, USA) was maintained at or above 40% of air saturation by adjusting the agitation speed up to 800 rpm or by

automatically supplementing air with pure oxygen using computer-controlled mass flow controllers. The total gas flow was kept at 2 l/min. CO_2 in the exit gas was measured with an infrared CO_2 monitor (Guardian Plus, Topac Inc. Hingham, MA, USA). Feed medium addition was computer-controlled based on weight using an Ohaus GT 8000 balance (Pine Brook, NJ, USA) and a Cole-Parmer (Vernon Hills, IL, USA) peristaltic pump.

The HCDC fermentation was done in three stages (modified from López-Cuellar et al. 2011):

Stage 1: Initial batch cultivation.

Stage 2: Exponential fructose feeding at a predetermined specific growth rate (μ) of 0.15 and $Y_{x/fructose}$ of 0.44 g/g to achieve a high density culture (Sun et al. 2006).

Stage 3: PHA accumulation using canola oil with N-limitation: 150 g of canola oil was added at 3 h intervals for a total of 450 g.

For the transformant *C. necator* pMPJAS03, fermentations were carried out in the similar manner as mentioned above and in addition arabinose (0, 0.001, 0.01, or 0.1 %) (Fukui et al. 2002; Sun et al. 2006) was added at the start of stage 3 to induce expression of $fadE_{Ec}$-$phaJ1_{Pp}$ which are under the control of $araC$-P_{BAD} promoter.

5.2.3 Analytical procedures

The dry cell weight (X) was measured gravimetrically. After the reactor sample was centrifuged at 14,500×*g* for 15 min at 4°C, the cells were washed with distilled water then lyophilized before weighing. Biomass samples with oil were washed twice with hexane and with distilled water then lyophilized before weighing. The supernatant was frozen until fructose, ammonium and phosphate analyses. Fructose was determined by the dinitrosalicyclic acid (DNS) method (Miller 1959). Ammonium was analyzed

according to Weatherburn (1967). Phosphate was measured via reduction of phosphomolybdate to molybdene blue (Clesceri et al. 1999). Canola oil was quantified gravimetrically and by fatty acid methyl ester analysis using GC-FID (Ichihara and Fukubayashi 2010).

5.2.4 PHA content and composition

5.2.4.1 GC-FID

PHA content and composition was determined as described by Sun et al. (2007). Briefly, to the lyophilized biomass or extracted PHA, 2 ml of methanol acidified with 15 % (v/v) H_2SO_4 containing 0.2 % (w/v) of benzoic acid (internal standard) and 2 ml of chloroform were mixed in a screw-capped tube and heated at 100°C for 3.5 h. After cooling, 2 ml of distilled water was added to the mixture to induce phase separation and the organic layer was recovered for GC-FID analysis.

5.2.4.2 Monomer composition determination by GC-MS

Monomer methyl esters were identified with a Varian CP 3800 Gas Chromatograph equipped with a Factor four capillary 5 MS column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness). Mass data were acquired and processed with a 1200 Quadrupole mass spectrometer. Sample (1 µl) in organic phase was injected with helium as the carrier gas, and the oven temperature was programmed at 80°C for 4 min, then increased at 8°C/min to 220°C, then 20°C/min to 260°C. The injector temperature was 250°C. Spectra were obtained as electron impact with an ionizing energy 70 eV.

5.3 Characterization of polyhydroxyalkanoates

5.3.1 PHA extraction for thermomechanical property assessment

To obtain PHA for thermal and mechanical properties determination, the biomass at the end of the fermentation process was centrifuged at 5,000×*g* for 30 min, washed twice with hexane followed by centrifugation then washed with distilled water, recentrifuged and lyophilized. Chloroform was added to the lyophilized biomass and agitated overnight. PHA was recovered from the chloroform solution after filtration and precipitation with three times the volume of ice cold methanol by drop wise addition. All samples were solubilized and precipitated with methanol twice. After the second precipitation, the polymer was recovered by filtration and left at room temperature for any residual solvent to evaporate.

5.3.2 Differential scanning calorimetry

The T_m, T_g and heat of fusion (ΔH_m) of purified PHA and PHB samples were obtained by differential scanning calorimetry (DSC) using a TA Instruments DSC Q100. Pure PHB (Biomer, Krailling, Germany) was used for comparison. Since mcl PHAs crystallize slowly, the samples were preconditioned to eliminate their thermal history by melting them at 175°C in an oven and allowing them to crystallize at room temperature for five days prior to analysis. The conditioned samples were sealed in aluminium pans, equilibrated at -70°C, kept isothermally for 5 min and were subsequently heated from -70 to 185°C at a rate of 5°C/min and kept at 185°C for 5 min before cooling to -70°C at a rate of 10°C/min. The samples were maintained at -70°C for 5 min and reheated to 185°C/min at a rate of 5°C/min. The T_m was taken as the peak of the endothermic curve of the first heating cycle, whereas the crystallinity was calculated from the heat of fusion

obtained from the 1st heating endotherm (Laycock et al. 2012). The % crystallinity of the polymers, X_c, was estimated using the following equation.

$$X_c = \frac{\Delta H_m}{\Delta H_{100}} \times 100$$

where ΔH_m is the apparent fusion enthalpy and ΔH_{100} is the theoretical fusion enthalpy of a 100 % crystalline polymer, which is 146 J/g (Barham et al. 1984).

5.3.3 Mechanical properties of PHAs

Solvent extracted scl-mcl PHA pellets and PHB (BIOMER, Krailling, Germany) were pre-dried in vacuum oven at 100°C for three hours before being compression molded in a Carver press at 190 ± 5°C with a 3 min residence time under 5000 N force then quenched in cold water. The standard type V test specimens were stamped from the compression molded sheets.

Tensile tests were conducted according to ASTM D638 for type V test specimens, using an Instron 3369 Universal tester at a cross head speed (CHS) of 5 mm/min. The average of five measurements is reported.

6 Results

6.1 Cloning and co-expression of acyl-CoA dehydrogenase ($fadE_{Ec}$) and enoyl-CoA hydratase ($phaJ1_{Pp}$) in *C. necator* pMPJAS03

In the present study, acyl-CoA dehydrogenase ($fadE_{Ec}$) and enoyl-CoA hydratase ($phaJ1_{Pp}$) were selected to be co-expressed under the inducible $araC\text{-}P_{BAD}$ promoter to increase the mcl content of scl-mcl PHA (Figure 6.1).

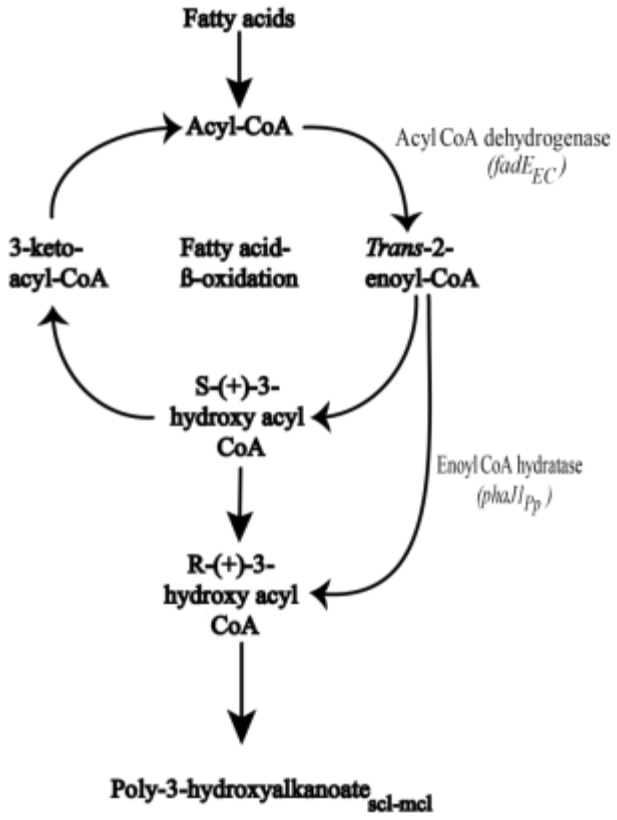


Figure 6.1: Proposed fatty acid β oxidation pathway in *C. necator* pMPJAS03 from acyl-CoA dehydrogenase ($fadE_{Ec}$) and enoyl-CoA hydratase ($phaJ1_{Pp}$) to enhance the incorporation of canola derived mcl monomers into scl-mcl PHA copolymer. Modified from Steinbüchel (2001)

6.1.1 Screening and confirmation of plasmid pMPJAS01

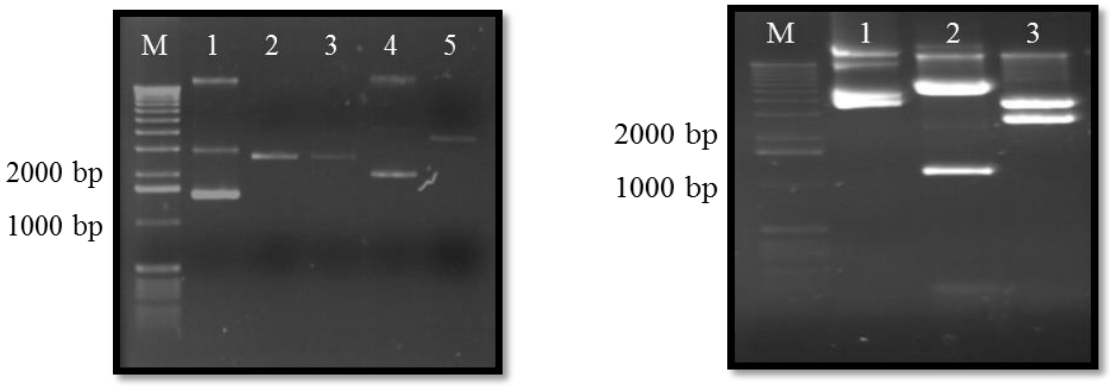


Figure 6.2: (A) Restriction digestion of pK18 and pBTB-3. (B) Verification of pMPJAS01 orientation.

(A) Restriction digestion of pK18 and pBTB-3. Lane M - DNA Marker; Lane 1 - pK18 control DNA; Lane 2 & 3 - pK18 linearized with Hind III (2661 bp); Lane 4 - pBTB-3 control DNA; Lane 5 - pBTB-3 linearized with Hind III (3586 bp);

(B) Verification of pMPJAS01 orientation. Lane M - DNA Marker; Lane 1 - Control DNA; Lane 2 - BamHI (4918 bp, 1214 bp, 115 bp); Lane 3 - HindIII (3586 bp, 2661 bp);

The plasmids pBTB-3 (3586 bp) and pK18 (2661 bp) were ligated together into their respective HindIII sites to form plasmid pMPJAS01 (6247 bp). The resultant plasmid was then digested with BamHI or HindIII to confirm the orientation of the plasmid as shown in Figure 6.2.

6.1.2 Screening and confirmation of plasmid pMPJAS02

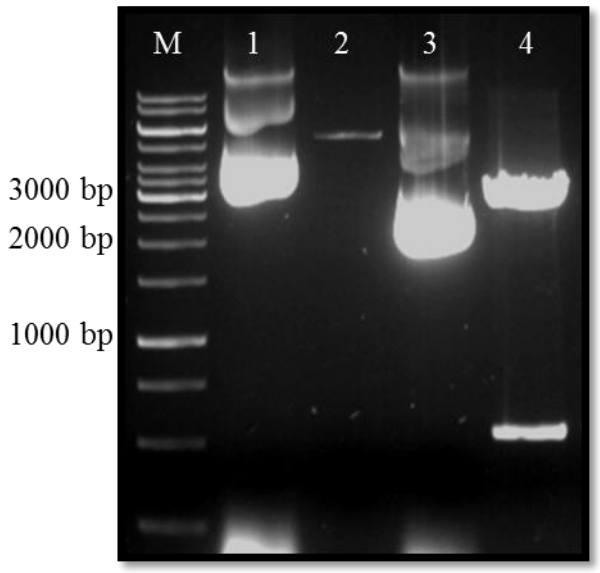


Figure 6.3: Restriction digestion of GS4560-1 pBSk Gene 1 (*fadE*$_{Ec}$) and GS4560-2 pBSk Gene 2 (*phaJ1*$_{Pp}$).

Lane M - DNA Marker; Lane 1 - GS4560-1 pBSk Gene 1 (*fadE*$_{Ec}$) control DNA; Lane 2 - GS4560-1 pBSk Gene 1 (*fadE*$_{Ec}$) linearized with HindIII (5346 bp); Lane 3 - GS4560-2 pBSk Gene 2 (*phaJ1*$_{Pp}$) control DNA; Lane 4 - GS4560-2 pBSk Gene 2 (*phaJ1*$_{Pp}$) digestion with EcoRV + XhoI (516 bp);

The Klenow blunted EcoRV + XhoI fragment of the plasmid GS45640-1 pBSK gene 2 harboring the *phaJ1*$_{Pp}$ was ligated into the HindIII linearized and blunted plasmid GS45640-1 pBSK gene 1 (*fadE*$_{Ec}$) to form pMPJAS02 (Figure 6.3 & 6.4).

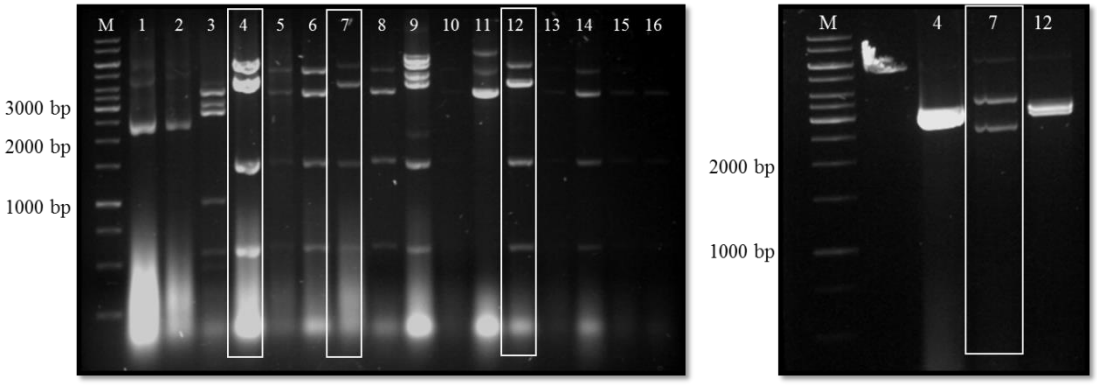


Figure 6.4: (A) Screening and restriction digestion of pMPJAS02. (B) Verification of pMPJAS02 orientation with restriction enzyme - HincII.

(A) Screening and restriction digestion of pMPJAS02. Lane M - Marker DNA; Lane 1-16: Agarose gel screening of plasmid DNA from the clones of pMPJAS02 digested with NcoI + AccI; Lane 4, 7 & 12 – Identification of positive clones with NcoI + AccI (3866, 1429, 567 bp) digestion;

(B) Verification of pMPJAS02 orientation with restriction enzyme – HincII. Lane M- Marker DNA; Clone 7 shows the positive orientation (3375, 2487 bp); Clone 4 & 12 shows the negative orientation (2977, 2800 bp);

6.1.3 Screening and confirmation of plasmid pMPJAS03

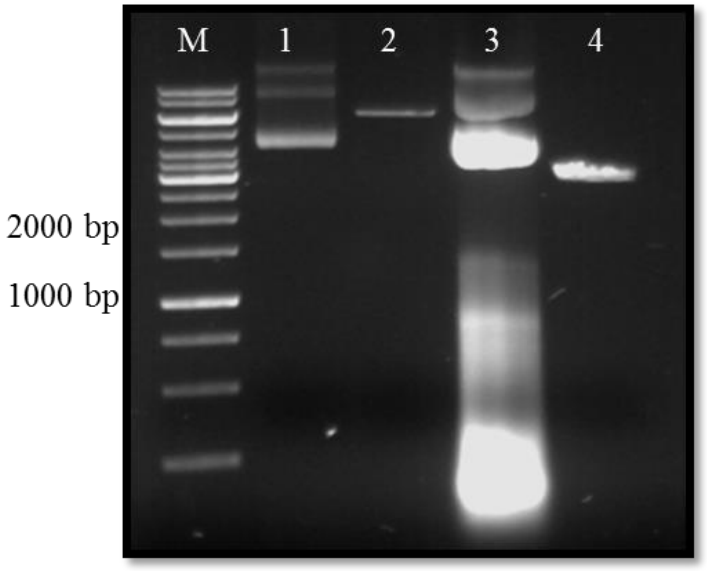


Figure 6.5: Restriction digestion of pMPJAS01 and pMPJAS02.

Lane M - Marker DNA; Lane 1 - pMPJAS01 control DNA; Lane 2 - pMPJAS01 linearized with SmaI digestion; Lane 3 - pMPJAS02 control DNA; Lane 4 - pMPJAS02 digestion with AccI + Hind III showing the 3.0 Kb $fadE_{Ec}$- $phaJ1_{Pp}$ fragment;

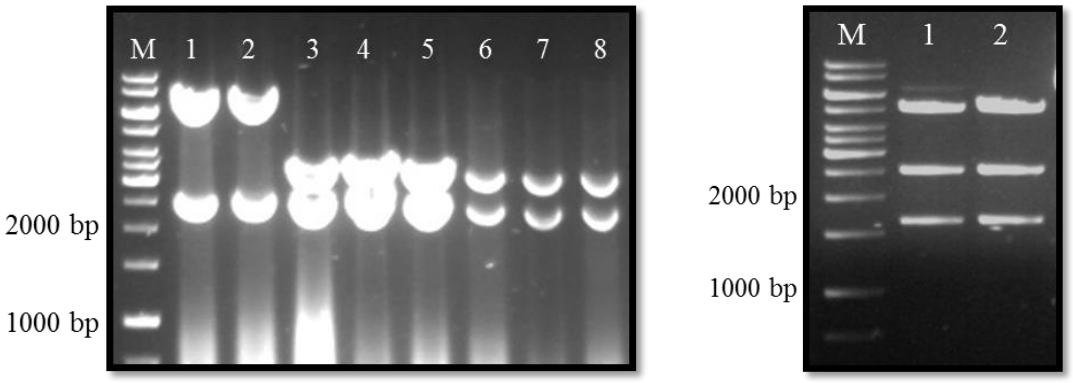


Figure 6.6: (A) Screening and restriction digestion of pMPJAS03. (B) Verification of pMPJAS03 orientation.

(A) Screening and restriction digestion of pMPJAS03. Lane M - Marker DNA; Lane 1-8: Agarose gel screening of plasmid DNA from the clones of pMPJAS03 digested with EcoRV; Lane 1 & 2: Positive clones with EcoRV (6606, 2640 bp) digestion;

(B) Verification of pMPJAS03 orientation. Lane M- Marker DNA; Lane 1 & 2: - pMPJAS03 digested with Hinc II + AclI (5055, 2506, 1685 bp) shows the positive orientation;

6.1.4 Isolation and verification of plasmid pMPJAS03 obtained from the clones of *C. necator* through electroporation process

The AccI + HindIII blunt 3.0 kb *fadE*$_{Ec}$-*phaJ1*$_{Pp}$ DNA fragment from pMPJAS02 was ligated to the SmaI linearised pMPJAS01 to form pMPJAS03. The plasmid pMPJAS03 was then electroporated into *C. necator* (Figure 6.5 to 6.7).

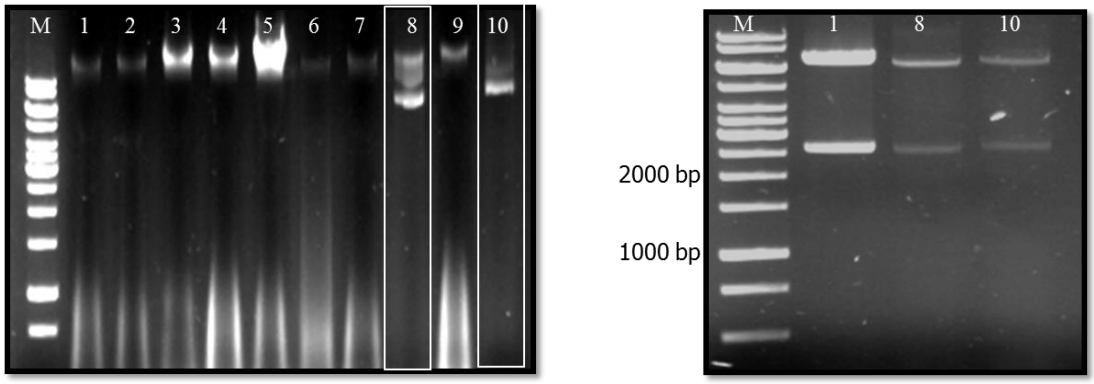


Figure 6.7: (A) Agarose gel showing the isolation of pMPJAS03 plasmid DNA obtained from the clones of *C. necator* through electroporation process. (B) Verification of pMPJAS03 with EcoRV digestion.

(A) Agarose gel showing the isolation of pMPJAS03 plasmid DNA obtained from the clones of *C. necator* through electroporation process. Lane M - Marker DNA; Lane 1-10 – Isolation of pMPJAS03 plasmid DNA from *C. necator* clones.

(B) Verification of pMPJAS03 with EcoRV digestion. Lane M - Marker DNA; Lane 1 - Control DNA; Clone 8 & 10 - Positive clones;

6.2 Increased production of scl-mcl PHA in wild-type *C. necator*

6.2.1 High cell density culture of *C. necator* H16

Typical results of several fermentations conducted to evaluate the three-stage feeding strategy are shown in Figure 6.8. The batch cultivation (stage 1) lasted 8 h with *C. necator* using the 3 g/l of fructose initially present in the bioreactor. Exponential fructose feeding at a predetermined μ of 0.15 h^{-1} (stage 2) began automatically and virtually all fructose was consumed until 30 h. Feeding at a higher rate led to accumulation of fructose: after 30 h of feeding at the same μ, the fructose feed rate was around 10 g/l·h and resulted in a slight accumulation of fructose. Exponential feeding continued until 39.9 h when the fructose feed rate was 15.5 g/l·h and the maximum carbon dioxide production rate (CPR) was 36 g/h (10.4 g/l·h). When the fructose feeding was stopped to allow the residual fructose in the fermenter to be consumed, the reduction in reactor fructose concentration corresponded to a decrease in CPR. At the end of the fed batch stage, the reactor fructose concentration was measured as 2 g/l. Later fructose measurement was not feasible due to the interference of canola oil in the analysis. The concentration of NH_4^+ was maintained between 1.20 to 1.35 mg/ml. Although a significant amount of phosphate precipitated at the start of the fermentation, it later redissolved and a concentration of ~ 2.0 g/l was measured at the end of the fermentation. A total of 44.4 g biomass/l was produced with an accumulation of 20 % w/w of PHB. The experimental $Y_{x/fructose}$ during stage 2 was determined to be 0.44 g/g and is the same value as entered in the feeding control program where it was required to predict the growth rate. The DO decreased to 40 % at this time and was maintained at this level for the rest of the process.

At the end of stage 2, canola oil was added three times at 3 h interval to avoid foaming. By 40-42 h, a small increase in CPR coincided with the consumption of the residual ammonium leading to N-limited conditions which favor PHA accumulation. During PHA accumulation on canola oil, the CPR was 2.0 to 2.5 g/h (0.5 to 0.7 g/l·h). About 5 % of all the canola oil added remained unused at the end of stage 3. The three stages totaled 94 h to produce 92 g/l of biomass containing 48 % PHA. (Figure 6.8).

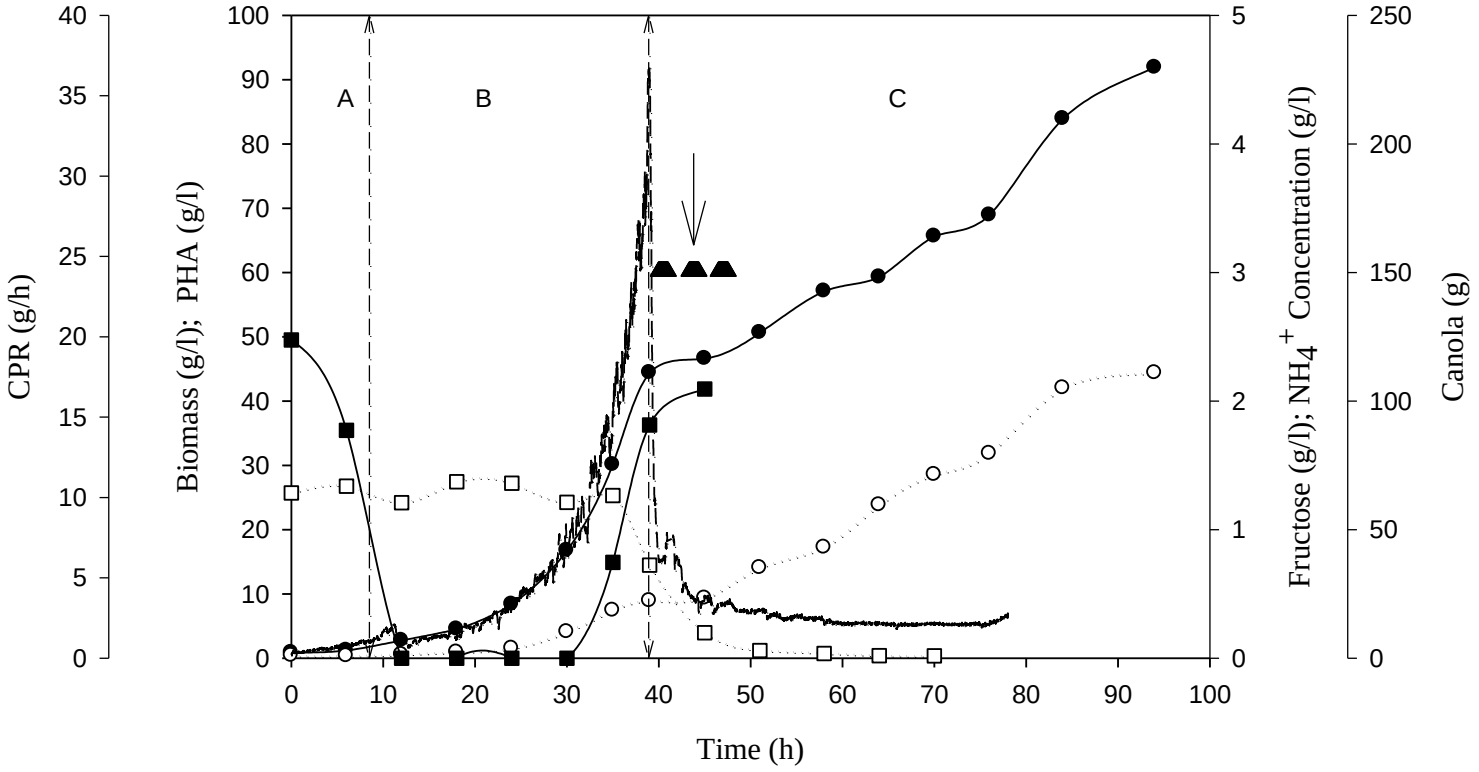


Figure 6.8: Higher biomass production of *C. necator* H16.

(A). Batch cultivation (Stage 1); (B). Fed batch - Exponential fructose feeding at $\mu = 0.15\ h^{-1}$ (Stage 2); (C). PHA accumulation - N-limited with canola oil added (Stage 3); fructose in reactor (■), NH_4^+ (□), biomass (●), PHA (○), CPR (---) and canola oil added (▲) where indicated by downward arrow. All data are the mean of two independent samples with a standard error of < 2 %.

6.2.2 PHA composition and thermomechanical properties

The monomeric composition of the scl-mcl-PHA was determined by GC-MS analysis with known PHA standards. The polymer was extracted with chloroform and precipitated twice in methanol to remove any 3-hydroxy carboxylic acids which may not have been associated with the polymer but not actually polymerized. Since these acids are soluble in methanol, they can be efficiently separated from methanol precipitated polymeric PHA by this method. After each chloroform extraction, the same peaks were present at similar relative intensities (Figure 6.9). If the minor peaks were from residual canola oil or unpolymerized 3-hydroxy acids, their relative intensities with respect to benzoic acid (the internal standard) would decrease with each extraction. The monomers in the scl-mcl PHA were identified by retention time and GC/MS fragmentation patterns when compared to well characterized standards (Table 6.1). The retention times were close and the fragmentation patterns of the monomers were identical for both standards and samples and the latter were consistent with literature values (He et al. 1998; Louie et al. 2000). After calculating the mole fraction of each monomer from the peak area of the ions, 3HB (99.81 moles %) was seen to be by far the most abundant monomer with the balance being 0.06 % 3HV, 0.09 % 3HHx, 0.04 % 3HO and trace amounts of an unidentified 3-hydroxy acid with more than eight carbons. The melting temperature (179°C), crystallinity (54 %), tensile stress (25.1 Mpa) and Young's modulus (698 Mpa) for pure PHB decreased to 176°C, 52 %, 19.1 Mpa and 443 Mpa respectively for *C. necator* PHA produced in the 3-stage process.

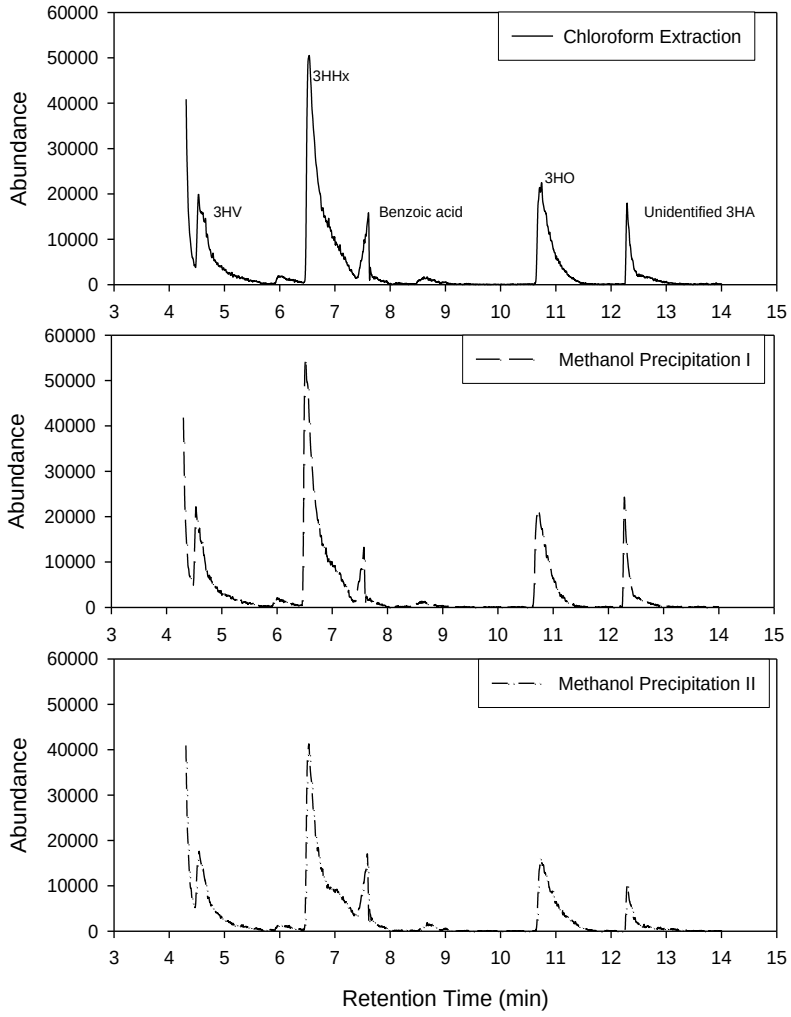


Figure 6.9: GC/MS analysis shows that the scl-mcl PHA has the same composition after chloroform extraction and after two successive methanol precipitations. Abundance (Y-axis) indicates flame ionization detector signal.

Table 6.1: GC MS analysis of PHA produced by *C. necator* using canola oil as carbon source

3-Hydroxyalkanoic acid methyl esters	Retention time (min)		m/z (EI)
	Standard	Sample	
3-hydroxybutyrate	3.05	3.15	71,74,103
3-hydroxyvalerate	4.60	4.54	59,74
3-hydroxyhexanoate	6.54	6.52	55,71,74,97,103,131,145
3-hydroxyoctanoate	11.0	10.75	55,71,74,100,103,104,156,173
Unidentified 3-hydroxyalkanoate	-	12.3	59, 101, 103, 156, 189

6.3 Co-expression of acyl-CoA dehydrogenase and enoyl-CoA hydratase for the production of scl-mcl PHA copolymer in *C. necator* pMPJAS03

6.3.1 High cell density cultivation of *C. necator* pMPJAS03

High cell density cultivation of *C. necator* pMPJAS03 was performed at various inducer concentrations. For each fermentation, there was a small difference in time switching from batch (stage 1) to fed-batch (stage 2) (indicated by period A in Figure 6.10) and from fed batch (stage 2) to PHA accumulation with canola oil (stage 3) (indicated by period B in Figure 6.10). This variation was due to differences in the lag phase, which affected the automated shift from batch to fed batch occurred and which was based on the detection of carbon limitation detected by a 25 % decrease in the carbon dioxide production rate (CPR). This resulted in minor differences in the duration of each fermentation which lasted 83-87 h.

The initial fructose concentration of around 2.5 g/l was consumed at the start of the batch stage and fructose did not begin to accumulate in the reactor till 30 hrs. However, by the end of stage 2, the fructose concentration was < 3 g/l. Accurate fructose measurement was not possible after oil addition. Using NH_4OH to control pH provided sufficient ammonium for growth. The reactor ammonium concentration was maintained at 1.0 to 1.4 mg/ml until N-limitation was imposed around 45 h (Figure 6.10). Phosphate concentration at the end of the fermentation was still 2.5 to 3.5 g/l.

The trend in the carbon dioxide production rate (CPR) was similar at all inducer concentrations (Figure 6.10). CPR was about 1.0 g/h at stage 1 and increased to 30 to 35 g/h during stage 2 at the end of which, it decreased to below 5 g/h. At this stage, the fructose concentration was < 3 g/l and was the appropriate time to add arabinose to induce the gene expression that were under the control of *araC*-P_{BAD} promoter. Following

induction, canola oil (150 g) was added in three successive additions at 3 h intervals resulting in a small increase in CPR as the residual ammonium was consumed leading to N-limitation for stimulation of PHA accumulation.

6.3.2 Biomass and PHA production without arabinose

Without arabinose, a maximum biomass of 96.4 g/l was obtained with *C. necator* pMPJAS03 in comparison to 91.9 g/l achieved with the wildtype using the same fermentation strategy. Similar levels of PHA of around 49.8 % and 48.3 % w/w were produced by the wildtype and transformant respectively. The monomeric composition of PHA produced by both bacteria was also similar, with 3HB being predominant with minor amounts of 3HV, 3HHx and 3HO. Additionally the transformant produced 3HD monomers. Although there were slight variations without the inducer, the transformant and wildtype had essentially similar growth and PHA composition. Most of the canola oil was utilized since there was only 5 % left at the end of the fermentation.

6.3.3 Effect of arabinose concentration on transformant

Increasing amounts of arabinose to induce gene expression had a profound effect on growth and PHA accumulation. As there was no difference in the fermentation strategy until arabinose was added at the start of the stage 3, cell growth, PHA production and residual biomass concentration were similar in all cases until this point. Both biomass and PHA production decreased with increasing arabinose concentration (Figure 6.10). The PHA monomers identified were 3HB, 3HV, 3HHx, 3HO and 3HD.

At the lowest inducer concentration (0.001 %), < 10 % of the canola oil was left unused as determined by GC-FID but at higher inducer concentrations (0.01 and 0.1 %) about 40% of canola oil remained as determined gravimetrically. No foaming was

observed in the absence or at low concentration (0.001 %) of the inducer but within 10 h of adding canola oil at 0.01 or 0.1 % arabinose, foaming (an indicator of cell death/lysis) occurred. A summary of the results of all the fermentation data is shown in Table 6.2 and is compared with the wildtype *C. necator* H16.

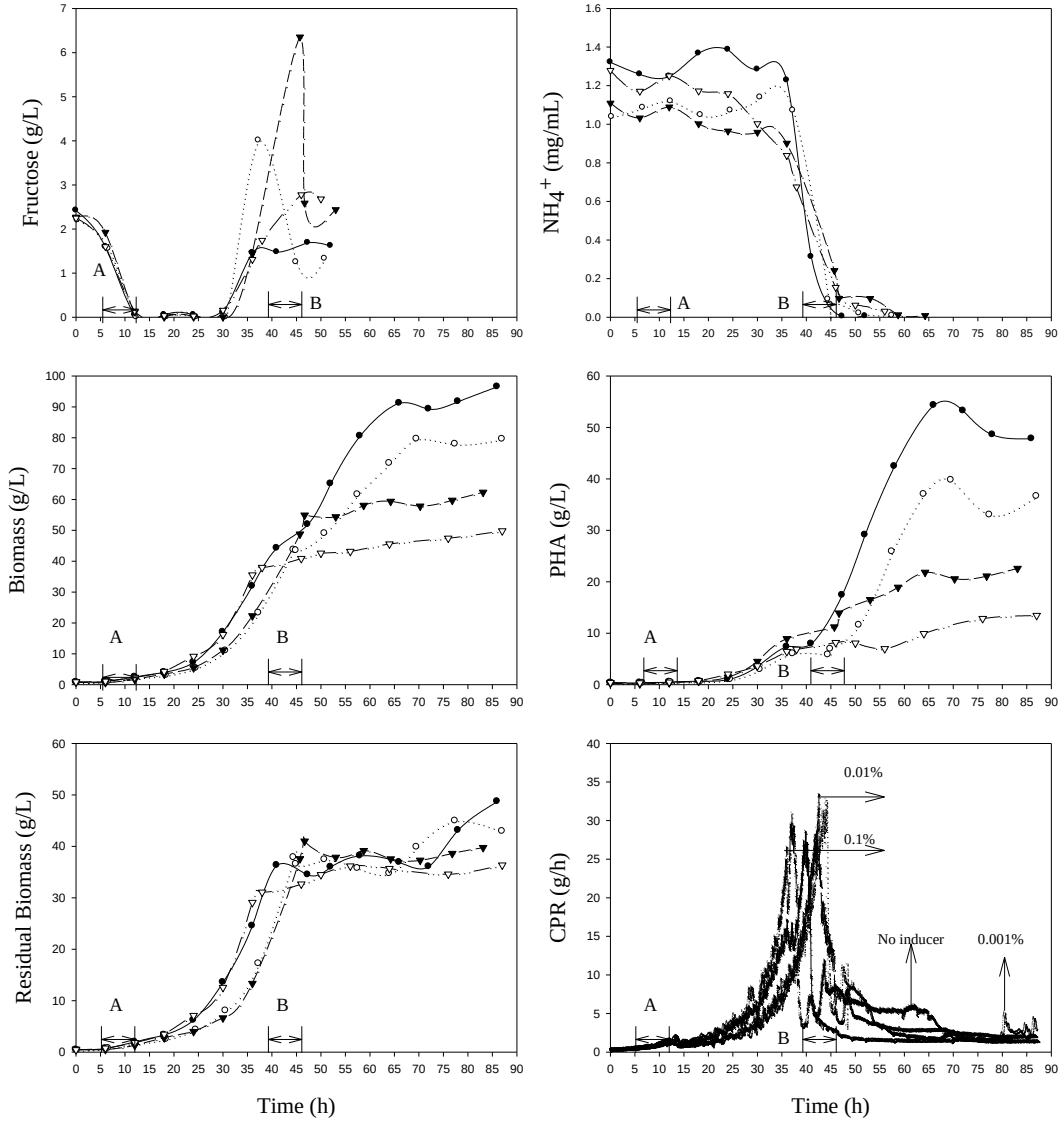


Figure 6.10: High cell density culture of *C. necator* pMPJAS03.

A 3-stage fermentation process in a 5 l fermenter with 0 % (●), 0.001 % (○), 0.01 % (▾) and 0.1 % (▽) arabinose as the inducer of PHA accumulation. The shift from batch to fed batch occurred during period (A) and from fed-batch to PHA accumulation on canola oil during period (B).

Table 6.2: Summary of fermentation data. With an exponential fructose feeding at μ = 0.15 h^{-1}. Arabinose inducer added at the end of the fed-batch stage with canola oil.

Microorganism	Inducer	Fed-batch stage			Overall process			Monomer composition of PHAs (mol %)					
		X	PHAs	Ended at	X	PHAs	Ended at	3HB	3HV	3HHx	3HO	Unidentified Peak	3HD
	(%)	g/l	% w/w	h	g/l	% w/w	h	3.1[a]	4.5[a]	6.5[a]	10.7[a]	12.3[a]	14.5[a]
C. necator H16	-	44.4	20.0	39.7	91.9	48.3	94	99.80	0.06	0.09	0.04	0.01	-
C. necator pMPJAS03	0	44.1	17.9	41.0	96.4	49.8	86	99.76	0.06	0.07	0.05	0.01	0.04
	0.001	43.4	15.8	44.9	79.5	46.1	87	99.75	0.07	0.10	0.05	0.02	0.02
	0.01	48.8	21.6	45.7	62.3	36.2	83	99.56	0.08	0.15	0.10	0.03	0.09
	0.1	37.9	18.1	38.0	49.8	27.0	87	99.67	0.09	0.16	0.05	0.01	0.01

[a] - GC/MS Retention time in minutes

6.4 Characterization of PHAs

There was a slight but steady increase in most monomers other than 3HB with increasing inducer concentration. The effects of their incorporation were evaluated by thermal and mechanical characterization.

6.4.1 Thermal properties

The T_m and crystallinity of scl-mcl PHAs produced by the wildtype and the transformant at different inducer concentrations followed a similar trend (Table 6.3). The T_m decreased from 179°C to 169°C as the amount of monomers other than 3HB increased from 0 for a PHB standard to 0.44 mol % mcl monomers while the crystallinity decreased from 54 % to 45 %.

6.4.2 Mechanical properties

The mechanical properties of scl-mcl PHA produced by *C. necator* H16 and *C. necator* pMPJAS03 at 0.01 % inducer concentration were compared with the pure PHB. Young's modulus, tensile stress and elongation at break values decreased with the increase of 3HA monomeric content other than 3HB (Table 6.3).

Table 6.3: Thermal and mechanical properties of scl-mcl PHA from *C. necator* H16 and from *C. necator* pMPJAS03.

Microorganism	Inducer (%)	T_m[a] °C	Crystallinity[a] %	Tensile stress[b] (MPa)	Young's Modulus[b] (MPa)	Extension at Break[b] (%)
Standard PHB		179	54	25.10 ± 1.0	698.00 ± 42.00	6.80 ± 0.90
C. necator H16	-	176	52	19.06 ± 1.19	442.68 ± 7.62	1.07 ± 0.15
C. necator pMPJAS03	No	177	54	-	-	-
	0.001%	174	53	-	-	-
	0.01%	169	45	10.64 ± 1.82	352.87 ± 12	Too Brittle
	0.1%	170	48	-	-	-

[a] – T_m and Crystallinity data are the mean of two independent samples. Standard error is < 2 %.

[b] – Tensile stress, Young's Modulus and extension at break data are the mean of five independent samples with standard deviation.

7 Discussion

Until recently (López-Cuellar et al. 2011), it has been reported that wildtype *C. necator* produces only homopolymeric PHB from vegetable oils (Fukui and Doi 1998; Kahar et al. 2004; Ng et al. 2010). It is likely that other researchers overlooked these monomers due to the relatively low resolution of GC-FID which is most commonly used for PHA analysis. Dennis et al. (1998) first demonstrated that the *C. necator* synthase could accept C6 substrates, while others have demonstrated that even larger monomers can be incorporated (Antonio et al. 2000). The present work shows that while mcl-PHA monomers can be produced, they are not preferred. For example, almost as much 3HV was produced as 3HHx even though canola contains only even carbon number carboxylic acids. Also substantially less 3HO was produced than 3HHx and vastly more 3HB was accumulated than any of the other monomers (Table 7.1).

Previous fermentation studies have demonstrated the production of homopolymeric PHB using either a sugar (Kim et al. 1994b; Ryu et al. 1999) or vegetable oil (Kahar et al. 2004) as the carbon source with wildtype *C. necator* H16 (Table 7.1). Much greater success has been obtained in producing PHB from glucose than from vegetable oil. Some strategies produced large amounts of biomass before inducing PHB synthesis by nutrient limitation. For example, Kim et al. (1994) determined that maximum PHB production could be achieved in their approach by initiating N-limitation around 70 g/l biomass (Table 7.1). Although, N-limitation was imposed at about 44 g/l biomass with fructose as the carbon source, PHB accumulation had already begun. However, since the amount of monomers other than 3HB was always very low, it is

likely that homopolymeric PHB and the scl-mcl PHA produced upon feeding with canola oil are miscible and would form a continuous phase upon processing.

Our data showed a great decrease in the carbon flux upon switching from fructose to canola oil. In contrast, Kahar et al. (2004) demonstrated that large amounts of P(3HB-3HHx) with 5 mol % 3HHx can be achieved by *C. necator* on soybean oil only, when a different PHA synthase is employed, and even better, if *phaJ* is cloned into this strain (Riedel et al. 2012). With wildtype *C. necator*, Kahar et al. (2004) reported just the PHB homopolymer being produced on soybean oil. It is thus clear that PHA accumulation in wildtype *C. necator* is not limited by lipase production nor by β-oxidation but rather by the supply of an easily utilizable substrate for the synthase. The only biochemical pathway available for carbon metabolism was PHA synthesis once nitrogen limitation was imposed. A bottleneck in that pathway, such as a synthase with a narrow substrate specificity, limited use of carbon.

In terms of fermentation substrates for the production of scl-mcl PHA, most studies have used commercially available fatty acids which are obtained by fractionation from vegetable oils. Carbon substrate cost can significantly contribute to the total production cost and may be as high as 40 % (Choi and Lee 1997). Given that the current commodity price for a metric ton of octanoic acid is $3.34K – 4.90K US (http://www.icispricing.com/il_shared/Samples/SubPage227.asp; http://www.alibaba.com/showroom/caprylic-acid-price.html) compared to soybean oil (US $1.12K) or canola oil (US $1.15K) (http://www.ers.usda.gov/data-products.aspx), PHA production cost may be reduced if the growth phase could be done using a carbohydrate substrate, as in this study. The cost of sugar substrates is significantly

lower, for example, the 2013 prices for dried high fructose corn syrup, specifically HFCS-42, and dried glucose syrup are US $639 and US $793 per metric ton (http://www.indexmundi.com/ commodities/), respectively. This paper clearly demonstrates the feasibility of scl-mcl-PHA production with a carbohydrate substrate like fructose being used for growth and canola oil for the accumulation phase in wildtype *C. necator*. However, this bacterium can typically accumulate in excess of 80 % PHA. López-Cuellar et al. (2011) produced biomass containing 90 % scl-mcl PHA. Achieving this percentage of PHA should lead to an economical commercial process providing that the material produced is of commercial value.

Thermomechanical analysis demonstrated that the incorporation of even a very small amount (less that 0.2 moles %) of mcl monomers can reduce melting point and crystallinity as well as substantially decreasing brittleness. However, the amount of mcl monomer should be further increased as the melting temperature was still high enough to cause difficulties (degradation) during melt processing. Inexpensive processes that can accomplish this should be investigated. For example, inhibition of β-oxidation can be used to further increase the mcl content (Green et al. 2002). Therefore, one approach to produce large quantities of PHA with higher mcl content using wildtype *C. necator* would be to inhibit β-oxidation while feeding both an alternative carbon and energy source, such as fructose, during the PHA accumulation phase. This should allow economical production of a more commercially useful material than homopolymeric PHB.

Table 7.1: Comparison of PHB and scl-mcl PHA production in *C. necator* and *E.coli*

Microorganism	Substrate	Control strategy	Fermentor volume l	Limitation	X g/l	PHA g/l	PHA %	Productivity g/l·h	PHA composition (mol %)							Reference
									3HB	3HV	3HHx	3HO	3HD	3HDD	MCL	
C. necator NCIMB 11599	Glucose	Online glucose analyzer	2.5	N	164	121	76	2.42	100	-	-	-	-	-	-	Kim et al. (1994b)
C. necator NCIMB 11599	Glucose	DO Stat	2.5	PO_4	210	175	83	3.02	100							Ryu et al. (1999)
	Glucose	Online glucose control	60	N	85	60	70	1.10	100	-	-	-	-	-	-	
	Glucose	DO Stat	60	PO_4	221	180	81	3.75	100							
C. necator H16	Soybean oil	Substrate concentration maintained at 20 g/l	10	PO_4	126	96	76	1.00	100	-	-	-	-	-	-	Kahar et al. (2004)
Recombinant C. necator Re2058/pCB113	Palm oil	Total substrate concentration 170 g/l	2	N	98	69	70	0.63	76	-	24	-	-	-	-	Riedel et al. (2012)
C. necator H16	Fructose & Canola oil	Fructose fed batch at a fixed flow rate of 0.9 l/h	5	N	20	19	92	0.43	95	-	-	-	-	-	5 [d]	López-Cuellar et al. (2011)
C. necator H16	Fructose & Canola oil	Exponential fructose feeding at μ 0.15	5	N	92	44	48	0.47	99.81	0.06	0.09	0.04	-	-	-	Present Study [e]
C. necator DSM 541 [a]	Fructose	Flask Study [c]	-	N	3.4	-	55	-	100	-	-	-	-	-	-	Dennis et al. (1998)
	Palmitate				0.51		58		93	1	6					
	Oleate				1.44		57		96	-	4					
E. coli LS 1298 [b]	Glucose	Flask Study	-	-	NA	-	67	-	100	-	nd	nd	nd	nd	-	Antonio et al.(2000)
	Octanoate						15		97.4	-	nd	2.6	nd	nd		
	Decanoate						15		98.6	-	nd	1.4	nd	nd		
	Dodecanoate						14		95	-	nd	1.5	nd	3.5		

NA - Not available; *nd* - not detectable.

[a] - Expression of *C. necator* acetoacetyl-CoA reductase and PHA-synthase ($phaC^+$, pha,A^-, $phaB^+$) under the control of *C. necator* σ^{70} promoter.

[b] - Expression of *C. necator phaCAB* operon under the control of lac promoter.

[c] - For the flask study, % PHA is in wt of polymer per ml of culture CDW.

[d] - Identified by ^1H and ^{13}C NMR analysis as 3HB, 3HV, 3HO, and 3HDD and 5 % of medium chain monomeric units with respect to 3HB.

[e] - Identified by GC-MS analysis. All data are the mean of two independent samples. Standard error is < 2 %.

C. necator H16 showed the accumulation of scl-mcl PHA copolymer by a HCDC process. In this, PHB was produced at the early stage in the fructose fed batch and other mcl monomer units accumulated during the canola oil stage. Therefore, we further extended our research to enhance the incorporation of canola derived mcl monomers into scl-mcl PHA copolymer production process by improving the oil metabolism using transformant *C. necator* pMPJAS03 with the co-expression of $fadE_{Ec}$ and $phaJ1_{Pp}$ genes under the control of $araC\text{-}P_{BAD}$ promoter system.

In the Table 7.2, a summary on the comparison of the scl-mcl PHA copolymer production by the engineered microorganisms on the metabolic pathways such as in the PHA biosynthesis pathway, fatty acid beta oxidation pathways or the enzymes linking both the pathways. Apart from engineering with the enzymes from the above pathways, PHA synthase presence in such construction plays a major role in the accumulation of scl-mcl PHA copolymer. Park et al. (2003) evaluated the effects of cloning $fadL_{Ec}$, $fadE_{Ec}$ and/or $fadD_{Ec}$ in FadA/FadB defective *E. coli* strains and reported that depending on the supplied fatty acid, enrichment of specific monomer composition was made by the additional expression of $fadL_{Ec}$ or $fadE_{Ec}$. Lu et al. (2003; 2004a; 2004b) cloned acyl-CoA dehydrogenase (*yafH*), enoyl-CoA hydratase with/without PHA synthase and found that the expression of *yafH* increased the cell dry weight and copolymer incorporation in the transformants. Fielder et al. (2002) co-expressed R-specific enoyl-CoA hydratase $phaJ_{Po/Pp}$ with the scl/mcl PHA synthase and yielded 3HB, 3HHx and 3HHx, 3HO, 3HD respectively. This study demonstrates that β-oxidation intermediates can be diverted to mcl PHA synthesis by the expression of R-specific enoyl-CoA hydratase. An interesting study from Sato et al. (2011) on the enoyl-CoA hydratase of *P. putida* KT2440 shows

that *phaJ1*$_{Pp}$ together with the PHA synthase accumulated mainly 3HHx and 3HO whereas the substrate specificity of *phaJ4*$_{Pp}$ contributed mainly to mcl PHA.

Co-expressing a gene from the β-oxidation pathway (*fadE*$_{Ec}$) and a gene linking β-oxidation with PHA biosynthesis pathway (*phaJ1*$_{Pp}$) in this study, yielded a small amount of 3HD monomers in the transformant which was not detected in wildtype. The result of Sato et al. (2011) coincides with our study stating that when *phaJ1*$_{Pp}$ is expressed, it mainly accumulates 3HHx and 3HO. Also, there has been an additional effect with the co-expression of *fadE*$_{Ec}$ which has contributed to the incorporation of 3HD units as mentioned by Park et al. (2003). The results of the HCDC with *araC-P*$_{BAD}$ expression system correlated well with the expression studies of Fukui et al. (2002) which demonstrated that at 0.001% arabinose concentration, only a trace amount or no incorporation of comonomer - 3HHx was noted. The optimal inducer concentration was 0.003-0.01%, as higher concentrations decreased the PHA content with the same amount of 3HHx. Arabinose is not metabolized as a carbon source for growth by *C. necator* (Fukui et al. 2011; Pohlmann et al. 2006). Therefore, L-arabinose enters the cell by an unknown transporter system and could be present intracellularly at an inhibitory concentration to the growth of *C. necator* (Fukui et al. 2011). Although the *P*$_{BAD}$ expression system is known for its tight regulation and control (Fukui et al. 2011; Fukui et al. 2002), when it comes to PHA accumulation in high cell density culture as presented in this work, the biomass production is poor at 0.1 % arabinose. Further, reduction in cell growth decreases the PHA production in this system thereby making this expression system less promising for polymer production.

Here, when attempting to express *fadE*$_{Ec}$ and *phaJ1*$_{Pp}$ yielded 3HB, 3HV, 3HHx, 3HO and 3HD in *C. necator* pMPJAS03. When compared with the other inducer concentration, 0.01% induction showed an increase in incorporation of 3HHx, 3HO and 3HD. Considering the application of *araC-P*$_{BAD}$ promoter system in the high cell density study, increasing amounts of arabinose to induce gene expression had a profound effect on growth and PHA accumulation. Therefore a suitable choice of positively regulated expression system should be employed to enhance the incorporation of mcl monomers into scl-mcl PHA copolymer in *C. necator*.

Although the high cell density production using the *araC-P*$_{BAD}$ promoter system was inadequate, the properties of the copolymer produced by the transformant *C. necator* with small amounts of mcl monomers are of potential interest. Scl PHA is a brittle polymer. Mcl PHAs on the other hand possess higher number of carbons in the side chain, making them elastic in nature. Addition of mcl units to 3HB resulted in a decrease in the heat of fusion and crystallinity, as shown in Table 6.2. This is a common phenomenon when an elastomeric material is added to a semi-crystalline one, because it disrupts the crystallization process. Furthermore, introduction of the mcl units lowered the melting temperature of 3HB as shown in Table 6.2. PHAs are prone to thermal degradation above 180°C and so a reduction in T_m by the incorporation of scl or mcl monomeric units can be beneficial, because it would widen the processability window. One way to achieve this is by incorporating scl units such as 3HV. These copolymers remain fragile and brittle because they provide a very small change in crystallinity (Noda et al. 2010). The other distinct groups of copolymers that demonstrate a decreasing trend in T_m are the copolymers of PHB with mcl PHA units such as 3HHx, 3HO and 3HD with

side groups of at least three carbons (Noda et al. 2010; Noda et al. 2005). As shown in the present work, these materials also show a reduction in crystallinity, which greatly depends on the polymer composition.

The incorporation of mcl unit in the 3HB polymer resulted in a significant reduction in Young's modulus and tensile strength. The decrease in tensile stress is typical of impact modification and can be justified by the decrease in crystallinity with the addition of mcl unit in the 3HB polymer and the introduction of a softer component. However, incorporation of mcl unit also caused a sharp drop in % elongation in *C.necator* H16 whereas in case of *C. necator* pMPJAS03 at 0.01 % inducer the test specimen produced were very fragile and brittle.

Table 7.2: Comparison of the β-oxidation genes involved in PHAs biosynthesis and copolymer production

Target organism	Gene manipulated	Other gene	Promoter	Carbon source	Monomer composition	Reference
E.coli WA101 (*fadA::Km*) *E.coli* WB101 (*fadB::Km*)	$fadE_{Ec}$	$phaC2_{Ps}$	*gntT104*	Sodium decanoate	3HHx,3HO,3HD	Park et al. (2003)
E.coli JM109, DH5α, XL1-Blue	$yafH_{Ec}$, $phaJ_{Ac}$	$phaC_{Ac}$	*Lac*	Lauric acid	3HB, 3HHx	Lu et al. (2003)
A. hydrophila CGMCC 0911-H	$yafH_{Ec}$	-	*Lac*	Lauric acid	3HB, 3HHx	Lu et al. (2004a)
E.coli XL1-Blue	$yafH_{Ec}$, $phaJ_{Ah}$	$phaC_{Ah}$	*Trc*	Lauric acid	3HB, 3HHx	Lu et al. (2004b)
E.coli JM109						
pSF6	$phaJ_{Po}$	$phaC_{Ap}$	*lac*	Decanoate	3HB, 3HHx	Fiedler et al. (2002)
pSF5	$phaJ_{Po}$	$phaC1_{Pa}$			3HHx,3HO,3HD	
pPS2, pSF7	$phaJ_{Pp}$	$phaC_{Ap}$			3HB, 3HHx	
pSF2, pSF7	$phaJ_{Pp}$	$phaC1_{Pa}$			3HHx,3HO,3HD	
E. coli LS5218	$phaJ1_{Pp}$ $phaJ4_{Pp}$	$phaC1_{Ps}$ $phaC1_{Ps}$	*Lac*	Sodium decanoate	3HB,3HHX,3HO,3HD,3HDD	Sato et al. (2011)
Recombinant *C. necator* PHB^{-}4	$phaJ_{Ac}$	ccr_{sc}, $phaC_{Ac}$	P_{BAD}	Fructose	3HB,3HHx	Fukui et al. (2002)
C. necator H16	-	-	-	Fructose & Canola Oil	3HB, 3HV, 3HHx, 3HO	Present study
C. necator H16 pMPJAS03	$fadE_{Ec}$, $phaJ1_{Pp}$	-	P_{BAD}	Fructose & Canola Oil	3HB, 3HV, 3HHx, 3HO, 3HD	Present study

Genes : *phaJ, phaJ1-Enoyl-CoA hydratase; phaC, phaC2-PHA synthase; CCR-Crotonyl-CoA reductase; fadE, yafH-acyl-CoA dehydrogenase;*

Abbreviated Microorganisms: *Ac-A. caviae; Ap- A. punctate; Cn- C. necator; Ec- E. coli; Pa- P. aeruginosa; Pp- P. putida; Po- P. oleovorans; Ps-Pseudomonas sp 61-3; Ra- R. aetherivorans 124; Sc- S. cinnamonensis;*

8 General conclusion and future work

8.1 Conclusion

The present study is an interdisciplinary approach to enhance the incorporation of canola derived mcl monomers into scl-mcl PHA copolymer using molecular biology, fermentation technology and material characterization studies in *C. necator*.

The current study proves that the wildtype *C. necator* H16 is capable of accumulating scl-mcl PHA copolymer at high cell density culture and from the *C. necator* metabolism, it was understood that a bottleneck in the PHA biosynthetic pathway, such as a synthase with narrow substrate specificity, limited use of carbon. To increase the incorporation of mcl monomer into scl-mcl PHAs, a recombinant *C. necator* harboring $fadE_{Ec}$ and $phaJ1_{Pp}$ under $araC\text{-}P_{BAD}$ promoter system was used in a high cell density culture. Thermomechanical analysis demonstrated that the incorporation of even a very small amount (less that 0.2 moles %) of mcl monomers can reduce melting point and crystallinity as well as substantially decreasing brittleness. However, the amount of mcl monomer should be further increased as the melting temperature was still high enough to cause difficulties (degradation) during melt processing.

The hypothesis of the study was proved from the analysis of the thermomechanical properties stating that the transformant *C. necator* pMPJAS03 was able to make a scl-mcl PHA copolymer with better thermal and mechanical properties than the wildtype *C. necator* H16. Henceforth, this initial research could pave way for the development and economical production of a more commercially useful material than homopolymeric PHB.

8.2 Future work

The study on HCDC of *C. necator* on fructose and canola oil was carried out for the first time and the organism was able to accumulate around 48-49 % w/w of PHAs using N-limitation. Therefore steps have to be taken to improve the % accumulation of PHA in order to improve the productivity of the process. This can be accomplished by imposing PO_4-limitation in the process.

In this study, canola oil was found to be metabolized well by the *C. necator* but the % mcl incorporation was found to be less than 0.1 % in both wildtype and the transformant. Therefore future studies can focus on adding suitable β oxidation inhibitors such as acrylic acid in the third stage of fermentation process, which would facilitate the availability of mcl precursors for improved incorporation of mcl PHAs.

Considering the application of *araC-*P_{BAD} promoter system in the high cell density study, increasing amounts of arabinose to induce gene expression had a profound effect on growth and PHA accumulation. Henceforth, further research should be carried out employing suitable positively regulated expression system to enhance the incorporation of mcl monomers into scl-mcl PHA copolymer in *C. necator* H16.

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ORIGINAL PAPER

A feeding strategy for incorporation of canola derived medium-chain-length monomers into the PHA produced by wild-type *Cupriavidus necator*

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Abstract The aim of this study was to increase the density of wild type *Cupriavidus necator* H16 biomass grown on fructose in order to produce sufficient copolymer of short-chain-length (scl) and medium-chain-length (mcl) polyhydroxyalkanoate (PHA) from canola oil for mechanical testing of the PHA. Initial batch cultivation on fructose was followed by exponential feeding of fructose at a predetermined μ to achieve 44.4 g biomass/l containing only 20 % w/w of polyhydroxybutyrate (PHB) with a $Y_{x/fructose}$ of 0.44 g/g. In a third stage, canola oil was added under N-limited conditions to produce 92 g/l of biomass with 48 % w/w scl–mcl PHA. Using known standards, the PHA composition was confirmed by GC–MS analysis as 99.81 % 3-hydroxybutyrate, 0.06 % 3-hydroxyvalerate, 0.09 % 3-hydroxyhexanoate and 0.04 % 3-hydroxyoctanoate. The melting temperature (179 °C), crystallinity (54 %), tensile stress (25.1 Mpa) and Young's modulus (698 Mpa) for a PHB standard decreased to 176 °C, 52 %, 19.1 and 443 Mpa respectively for *C. necator* PHA produced in the 3-stage process.

Keywords Polyhydroxyalkanoates · *Cupriavidus necator* · Fed-batch fermentation · Fructose · Canola oil

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Introduction

Polyhydroxyalkanoates (PHAs) are a range of polymers which can be synthesized by a variety of microorganisms as intracellular granules when growth is limited by an essential nutrient such as nitrogen or phosphorus in the presence of excess carbon source. PHAs can be divided into three main categories based on the number of carbons in the 3-hydroxyl fatty acid monomers: short-chain-length (scl) PHAs contain up to five carbons, medium-chain-length (mcl) PHAs have 6–14 carbons and long-chain-length (lcl) PHAs have more than 14 carbons (Madison and Huisman 1999).

The first PHA (i.e. poly-3-hydroxybutyrate (PHB)) was discovered and characterized by Lemoigne in the 1920s and found to be useful as a bioplastic by Baptiste in the 1950s. Its high melting point (near its degradation temperature) and low ductility prevented commercialization until methods were developed to feed suitable substrates to produce lower melting, less crystalline co-polymers. This was possible since the PHB synthase was found to accept slightly larger carbon substrates (C5 in addition to C4) and led to the commercialization of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(HB-HV)) by Imperial Chemical Industries. In the early 1980s, *Pseudomonas oleovorans* (now *putida*) and related organisms were discovered to produce another kind of PHA. This lower melting, elastomeric PHA could be accumulated from medium-chain-length carboxylic acids due to a different PHA synthase (Type II). It became doctrine that the original PHA synthase (Type I) could only accumulate C4 and C5 substrates (Haywood et al. 1989) until it was shown that the Type I synthases of *Rhodospirillum rubrum* (Brandl et al. 1989), *Thiocapsa pfennigii* (Libergesell et al. 1993) and *Aeromonas caviae* (Shimamura et al. 1994) could also

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polymerize significant amounts of C6 3-hydroxyalkanoates. This knowledge became of commercial importance when Proctor and Gamble patented scl–mcl PHAs under the trade name of NODAX (Noda 1996). It was believed that such materials could only be produced using special PHA synthases that could accumulate both scl and mcl substrates but it was eventually demonstrated that even the Type I synthase of *Ralstonia eutropha* (now *Cupriavidus necator)* could accept C6 and possibly larger substrates albeit with less affinity than some other synthases (Dennis et al. 1998). It was subsequently shown that, by manipulating the culture conditions (in this case inhibiting beta-oxidation), substantial amounts of 3-hydroxyhexanoate (HHx) could be incorporated into so-called scl-PHA using the *R. eutropha* synthase with sodium octanoate (Green et al. 2002).

More recently, it was demonstrated that less expensive substrates, plant oils, could be used by a bacterium with a Type I synthase to produce PHA with much lower crystallinity and melting point than was previously considered possible. Unfortunately, not enough polymer has been produced to determine the mechanical properties of these materials. López-Cuellar et al. (2011) has so far achieved the highest biomass production in a three stage process with most of the polymer accumulating in the third stage under N-limitation with canola oil as the carbon substrate. The copolymer was primarily HB and small amounts of HV, HO and HDD. However, only a maximum of 19 g/l of PHA was produced. There are no other reports on the bench scale production of scl–mcl PHA by *Cupriavidus necator* H16. In order to produce sufficient material for testing and to develop applications, the polymer must be produced to a higher level. While this bacterium has been successfully grown to high cell density using soybean oil as sole source of carbon, no mcl PHA was detected (Kahar et al. 2004). In other studies, glucose was the sole substrate used for the production of the PHB homopolymer (Kim et al. 1994; Ryu et al. 1999). Although carbohydrate substrates like glucose are less expensive than vegetable oils like soya bean oil, mcl PHAs have not been shown to be produced by wildtype *C. necator* from carbohydrates alone, and only López-Cuellar et al. (2011) has shown that scl–mcl PHA can be produced by wild type *C. necator* using fructose for growth and canola oil for polymer accumulation.

Exponential feeding at a defined μ has been demonstrated as an efficient automated feeding strategy that has been applied to the fed-batch cultivation of *P. putida* to produce mcl-PHA (Diniz et al. 2004; Sun et al. 2006). This approach can prevent the accumulation of inhibitory products or substrates (Riesenberg et al. 1991; Tada et al. 2000; Yoon et al. 1994) and has a low risk of overfeeding if done well below the maximum specific growth rate of the microorganism. For the above reasons, we evaluated the feasibility of producing higher biomass levels using the 3-stage process of López-Cuellar et al. (2011) but instead of linear feeding during the fed-batch stage, exponential feeding at a defined specific growth rate was used for the production of scl–mcl PHA by wild-type *C. necator*. The aim of this study was to improve upon the work of López-Cuellar et al. (2011) to produce sufficient material to allow thermomechanical testing of the type of PHA that it produces.

Materials and methods

Inoculum and growth media

Cupriavidus necator H16 (ATCC 17699, obtained from American Type Culture Collection, Manassas, VA) was maintained on nutrient agar plates at 4 °C. A 10 % (v/v) inoculum for the bioreactor was prepared in a medium containing per liter: 10 g fructose, 3.70 g $(NH_4)_2SO_4$, 0.40 g $MgSO_4$, 6.36 g $Na_2HPO_4{\cdot}7H_2O$, 2.70 g KH_2PO_4 and 1.00 g nutrient broth. The initial culture medium in the bioreactor contained per Liter: 3 g fructose, 4.70 g $(NH_4)_2SO_4$, 0.40 g $MgSO_4$, 7.7 g $Na_2HPO_4{\cdot}7H_2O$, 3.26 g KH_2PO_4 and 10 ml trace element solution. Each liter of trace element solution contained 10 g $FeSO_4{\cdot}7H_2O$, 3 g $CaCl_2{\cdot}2H_2O$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.15 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g $NiCl_2{\cdot}6H_2O$ and 1.00 g $CuSO_4{\cdot}5H_2O$. The feed medium for the fed-batch stage contained per Liter: 500 g fructose, 12 g $MgSO_4$ and 120 ml trace element solution. Canola oil was used as substrate for the PHA accumulation phase. During growth, 28 % (w/v) NH_4OH was used to control the pH and was replaced by 2 M KOH during the N-limited accumulation stage.

Fermentation studies

All fermentations were performed in a 5 l Minifors bioreactor (Infors HT, Bottmingen, Switzerland) at 30 ± 1 °C with a 3 l initial working volume. LabVIEW 6.1 (National Instruments, Austin, Texas) was used to monitor and/or control parameters such as dissolved oxygen (DO), outlet gas CO_2, carbon source feeding and pH. pH (Easyferm Plus K8200, Hamilton Company, USA) was maintained at 7.00 ± 0.05 by the addition of NH_4OH or KOH. DO (Mettler Toledo, Hamilton Company, USA) was maintained at or above 40 % of air saturation by adjusting the agitation speed up to 800 rpm or by automatically supplementing air with pure oxygen using computer-controlled mass flow controllers. The total gas flow was kept at 2 l/min. CO_2 in the exit gas was measured with an infrared

CO_2 monitor (Guardian Plus, Topac Inc. Hingham, MA, USA). Feed medium addition was computer-controlled based on weight using an Ohaus GT 8000 balance (Pine Brook, NJ, USA) and a Cole-Parmer (Vernon Hills, IL, USA) peristaltic pump.

The fermentation was done in three stages (modified from López-Cuellar et al. (2011)):

Stage 1: Initial batch cultivation.

Stage 2: Exponential fructose feeding at a predetermined specific growth rate (μ) of 0.15 as described by Sun et al. (2006) to achieve a high density culture.

Stage 3: PHA accumulation using canola oil with N-limitation: 150 g of canola oil was added at 3 h intervals for a total of 450 g.

Analytical procedures

The dry cell weight (X) was measured gravimetrically. After the reactor sample was centrifuged at 14,500×*g* for 15 min at 4 °C, the cells were washed with distilled water then lyophilized before weighing. Biomass samples with oil were washed twice with hexane and with distilled water then lyophilized before weighing. The supernatant was frozen until fructose, ammonium and phosphate analyses were performed. Fructose was determined by the dinitrosalicyclic acid (DNS) method (Miller 1959). Ammonium was analyzed according to Weatherburn (1967). Phosphate was measured via reduction of phosphomolybdate to molybdene blue (Clesceri et al. 1999). Fatty acid methyl esters were analyzed and compared to the standards by GC-FID (Ichihara and Fukubayashi 2010). PHA content and composition was determined as described by Sun et al. (2007). Briefly, to the lyophilized biomass or extracted PHA, 2 ml of methanol acidified with 15 % (v/v) H_2SO_4 containing 0.2 % (w/v) of benzoic acid (internal standard) and 2 ml of chloroform were mixed in a screw-capped tube and heated at 100 °C for 3.5 h. After cooling, 2 ml of distilled water was added to the mixture to induce phase separation and the organic layer was recovered for GC-FID analysis.

Monomer composition determination by GC–MS

Monomer methyl esters were identified with a Varian CP 3800 Gas Chromatograph equipped with a Factor four capillary 5 MS column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness). Mass data were acquired and processed with a 1200 Quadrupole mass spectrometer. Sample (1 μl) in organic phase was injected with helium as the carrier gas, and the oven temperature was programmed at 80 °C for 4 min, then increased at 8 °C/min to 220 °C, then 20 °C/min to 260 °C. The injector temperature was 250 °C. Spectra were obtained as electron impact with an ionizing energy 70 eV.

PHA extraction for thermomechanical property assessment

To obtain PHA for thermal and mechanical properties determination, the biomass at the end of the fermentation process was centrifuged at 5,000×*g* for 30 min, washed twice with hexane followed by centrifugation then washed with distilled water, recentrifuged and lyophilized. Chloroform was added to the lyophilized biomass and agitated overnight. PHA was recovered from the chloroform solution after filtration and precipitation with three times the volume of ice cold methanol by drop wise addition. All samples were solubilized and precipitated with methanol twice. After the second precipitation, the polymer was recovered by filtration and left at room temperature for any residual solvent to evaporate.

Differential scanning calorimetry

The melting temperature (T_m), glass transition temperature (T_g) and heat of fusion (ΔH_m) of purified PHA and PHB samples were obtained by differential scanning calorimetry (DSC) using a TA Instruments DSC Q100. Pure PHB (Biomer, Krailling, Germany) was used for comparison. Since MCL PHAs crystallize slowly, the samples were preconditioned to eliminate their thermal history by melting them at 175 °C in an oven and allowing them to crystallize at room temperature for 5 days prior to analysis. The conditioned samples were sealed in aluminium pans, equilibrated at −70 °C, kept isothermally for 5 min and were subsequently heated from −70 to 185 °C at a rate of 5 °C/min and kept at 185 °C for 5 min before cooling to −70 °C at a rate of 10 °C/min. The samples were maintained at −70 °C for 5 min and reheated to 185 °C/min at a rate of 5 °C/min. The T_m was taken as the peak of the endothermic curve of the first heating cycle, whereas the crystallinity was calculated from the heat of fusion obtained from the 1st heating endotherm (Laycock et al. 2013). The % crystallinity of the polymers, X_c, was estimated using the following equation

$$X_c = \frac{\Delta H_m}{\Delta H_{100}} \times 100$$

where ΔH_m is the apparent fusion enthalpy and ΔH_{100} is the theoretical fusion enthalpy of a 100 % crystalline polymer, which is 146 J/g (Barham et al. 1984).

Mechanical properties of PHAs

Solvent extracted scl–mcl PHA pellets and PHB (BIOMER, Krailling, Germany) were pre-dried in vacuum oven at 100 °C for 3 h before being compression molded in a Carver press at 190 ± 5 °C with a 3 min residence time

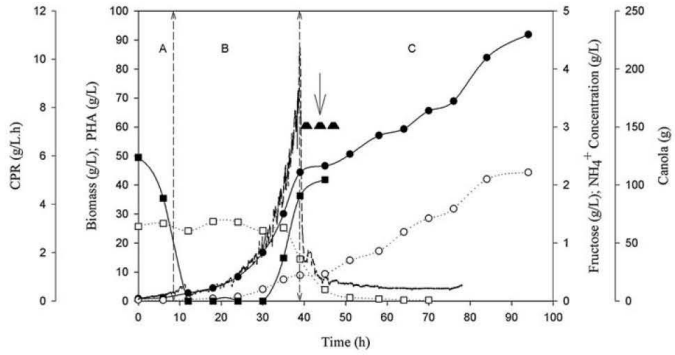

Fig. 1 Higher biomass production of *C. necator* H16. (*A*) Batch cultivation (Stage 1); (*B*) Fed batch—Exponential fructose feeding at $\mu = 0.15\ h^{-1}$ (Stage 2); (*C*) PHA accumulation—N-limited with canola oil added (Stage 3); fructose in reactor (*filled square*), NH_4^+ (*open square*), biomass (*filled circle*), PHA (*open circle*), CPR (*dashed line*) and canola oil added (*filled triangle*) where indicated by downward arrow. All data are the mean of two independent samples with a standard error of <2%

under 5,000 N force then quenched in cold water. The standard type V test specimens were stamped from the compression molded sheets.

Tensile tests were conducted according to ASTM D638 for type V test specimens, using an Instron 3369 Universal tester at a cross head speed of 5 mm/min. The average of five measurements is reported.

Results

High cell density culture of *C. necator* H16

Typical results of several fermentations conducted to evaluate the three-stage feeding strategy are shown in Fig. 1. The batch cultivation (stage 1) lasted 8 h with *C. necator* using the 3 g/l of fructose initially present in the bioreactor. Exponential fructose feeding at a predetermined μ of 0.15 h^{-1} (stage 2) began automatically and virtually all fructose was consumed until 30 h. Feeding at a higher rate led to accumulation of fructose. After 30 h of feeding at the same μ, the fructose feed rate was around 10 g/l h and resulted in a slight accumulation of fructose. Exponential feeding continued until 39.9 h when the fructose feed rate was 15.5 g/l h and the maximum carbon dioxide production rate (CPR) was 36 g/h (10.4 g/l/h). When the fructose feeding was stopped to allow the residual fructose in the fermenter to be consumed, the reduction in reactor fructose concentration corresponded to a decrease in CPR. At the end of the fed batch stage, the reactor fructose concentration was measured as 2 g/l. Later fructose measurement was not feasible due to the interference of canola oil in the analysis. The concentration of NH_4^+ was maintained between 1.20 and 1.35 mg/ml. Although a significant amount of phosphate precipitated at the start of the fermentation, it later redissolved and a concentration of ~2.0 g/l was measured at the end of the fermentation. A total of 44.4 g biomass/l was produced with an accumulation of 20 % w/w of PHB. The experimental $Y_{x/fructose}$ during stage 2 was determined to be 0.44 g/g and is the same value as entered in the feeding control program where it was required to predict the growth rate. The DO decreased to 40 % at this time and was maintained at this level for the rest of the process.

At the end of stage 2, canola oil was added three times at 3 h interval to avoid foaming. By 40–42 h, a small increase in CPR coincided with the consumption of the residual ammonium leading to N-limited conditions which favor PHA accumulation. During PHA accumulation on canola oil, the CPR was 2.0–2.5 g/h (0.5–0.7 g/l/h). About 5 % of all of the canola oil added remained unused at the end of stage 3. The 3 stages totaled 94 h to produce 92 g/l of biomass containing 48 % PHA (Fig. 1).

PHA composition and thermomechanical properties

The monomeric composition of the scl–mcl–PHA was determined using GC–MS analysis with known PHA standards. The polymer was extracted with chloroform and precipitated twice in methanol to remove any 3-hydroxy carboxylic acids which may not have been associated with the polymer but not actually polymerized. Since these acids are soluble in methanol, they can be efficiently separated from methanol precipitated polymeric PHA by this method. After each chloroform extraction, the same peaks were present at similar relative intensities (Fig. 2). If the minor peaks were from residual canola oil or unpolymerized 3-hydroxy acids, their relative intensities with respect to benzoic acid (the internal standard) would decrease with each extraction. The monomers in the scl–mcl PHA were identified by retention time and GC/MS fragmentation patterns when compared to well characterized standards (Table 1). The retention times were close and the fragmentation patterns of the monomers were identical for both

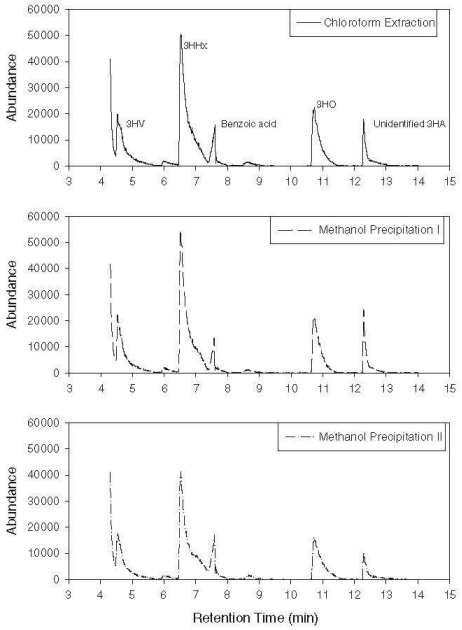

Fig. 2 GC/MS analysis shows that the scl–mcl PHA has the same composition after chloroform extraction and after two successive methanol precipitations. Abundance (Y-axis) indicates flame ionization detector signal

standards and samples and the latter were consistent with literature values (He et al. 1998; Louie et al. 2000). After calculating the mole fraction of each monomer from the peak area of the ions, HB (99.81 mol%) was seen to be by far the most abundant monomer with the balance being 0.06 % HV, 0.09 % HHx, 0.04 % HO and trace amounts of an unidentified 3-hydroxy acid with more than eight carbons. The melting temperature (179 °C), crystallinity (54 %), tensile stress (25.1 Mpa) and Young's modulus (698 Mpa) for pure PHB decreased to 176 °C, 52 %, 19.1 and 443 Mpa respectively for *C. necator* PHA produced in the 3-stage process.

Discussion

Until recently (López-Cuellar et al. 2011), it has been reported that wild type *C. necator* produces only homopolymeric PHB from vegetable oils (Fukui and Doi 1998; Kahar et al. 2004; Ng et al. 2010). It is likely that other researchers overlooked these monomers due to the relatively low resolution of GC-FID which is most commonly used for PHA analysis. Dennis et al. (1998) first demonstrated that the *C. necator* synthase could accept C6 substrates, while others have demonstrated that even larger monomers can be incorporated (Antonio et al. 2000). The present work shows that while mcl-PHA monomers can be produced, they are not preferred. For example, almost as much 3HV was produced as 3HHx even though canola contains only even carbon number carboxylic acids. Also substantially less 3HO was produced than 3HHx and vastly more 3HB was accumulated than any of the other monomers (Table 2).

Previous fermentation studies have demonstrated the production of homopolymeric PHB using either a sugar (Kim et al. 1994; Ryu et al. 1999) or vegetable oil (Kahar et al. 2004) as the carbon source with wild type *C. necator* H16 (Table 2). Much greater success has been obtained in producing PHB from glucose than from vegetable oil. Some strategies produced large amounts of biomass before inducing PHB synthesis by nutrient limitation. For example, Kim et al. (1994) determined that maximum PHB production could be achieved in their approach by initiating N-limitation around 70 g/l biomass (Table 2). Although we imposed N-limitation at about 44 g/l biomass with fructose as the carbon source, PHB accumulation had already begun. However, since the amount of monomers other than HB was always very low, it is likely that homopolymeric PHB and the scl-mcl PHA produced upon feeding with canola oil are miscible and would form a continuous phase upon processing.

Our data showed a great decrease in the carbon flux upon switching from fructose to canola oil. In contrast, Kahar et al. (2004) demonstrated that large amounts of P(3HB-3HHx) with 5 mol% 3HHx can be achieved by *C. necator* on soybean oil only, when a different PHA synthase is employed, and even better, if *phaJ* is cloned

Table 1 GC–MS analysis of PHA produced by *C. necator* using canola oil as carbon source

3-Hydroxyalkanoic acid methyl esters	Retention time (min)		m/z (EI)
	Standard	Sample	
3-hydroxybutyrate	3.05	3.15	71,74,103
3-hydroxyvalerate	4.60	4.54	59,74
3-hydroxyhexanoate	6.54	6.52	55,71,74,97,103,131,145
3-hydroxyoctanoate	11.0	10.75	55,71,74,100,103,104,156,173
Unidentified 3-hydroxyalkanoate	–	12.3	59, 101, 103, 156, 189

Table 2 Comparison of PHB and scl–mcl PHA production in *C. necator* and *E.coli*

Microorganism	Substrate	Control strategy	Fermentor volume l	Limitation	X (g/l)	PHA (g/l)	PHA %	Productivity (g/l h)	PHA composition (mol%)							Reference
									3HB	3HV	3HHx	3HO	3HD	3HDD	MCL	
C. necator NCIMB 11599	Glucose	Online glucose analyzer	2.5	N	164	121	76	2.42	100	–	–	–	–	–	–	Kim et al. (1994)
C. necator	Glucose	DO Stat	2.5	PO_4	210	175	83	3.02	100							
NCIMB 11599	Glucose	Online glucose control	60	N	85	60	70	1.1	100	–	–	–	–	–	–	Ryu et al. (1999)
	Glucose	DO Stat	60	PO_4	221	180	81	3.75	100							
C. necator H16	Soybean oil	Substrate concentration maintained at 20 g/l	10	PO_4	126	96	76	1.00	100	–	–	–	–	–	–	Kahar et al. (2004)
Recombinant C. necator Re2058/ pCB113	Palm oil	Total substrate concentration 170 g/l	2	N	98	69	70	0.63	76	–	24	–	–	–	–	Riedel et al. (2012)
C. necator H16	Fructose and Canola oil	Fructose fed batch at a fixed flow rate of 0.9 l/h	5	N	20	19	92	0.43	95	–	–	–	–	–	5[d]	López-Cuellar et al. (2011)
C. necator H16	Fructose and Canola oil	Exponential fructose feeding at μ 0.15	5	N	92	44	48	0.47	99.81	0.06	0.09	0.04	–	–	–	This Work[e]
C. necator	Fructose				3.4		55		100	–	–					
DSM 541[a]	Palmitate	Flask Study[c]	–	N	0.51	–	58	–	93	1	6	–	–	–	–	Dennis et al. (1998)
	Oleate				1.44		57		96	–	4					
E. coli LS 1298[b]	Glucose						67		100	–	nd	nd	nd	nd		
	Octanoate	Flask Study	–	–	NA	–	15	–	97.4	–	nd	2.6	nd	nd	–	Antonio et al. (2000)
	Decanoate						15		98.6	–	nd	1.4	nd	nd		
	Dodecanoate						14		95	–	nd	1.5	nd	3.5		

NA Not available, *nd* not detectable

[a] Expression of *C. necator* acetoacetyl-CoA reductase and PHA-synthase ($phaC^+$, $phaA^-$, $phaB^+$) under the control of *C. necator* σ^{70} promoter

[b] Expression of *C. necator phaCAB* operon under the control of lac promoter

[c] For the flask study, % PHA is in wt of polymer per ml of culture CDW

[d] Identified by ^1H and ^{13}C NMR analysis as 3HB, 3HV, 3HO, and 3HDD and 5 % of medium chain monomeric units with respect to 3HB

[e] Identified by GC–MS analysis. All data are the mean of two independent samples. Standard error is <2 %

into this strain (Riedel et al. 2012). With wildtype *C. necator*, Kahar et al. (2004) reported just the PHB homopolymer being produced on soybean oil. It is thus clear that PHA accumulation in wildtype *C. necator* is not limited by lipase production nor by β-oxidation but rather by the supply of an easily utilizable substrate for the synthase. The only biochemical pathway available for carbon metabolism was PHA synthesis once nitrogen limitation was imposed. A bottleneck in that pathway, such as a synthase with a narrow substrate specificity, limited use of carbon.

In terms of fermentation substrates for the production of scl–mcl PHA, most studies have used commercially available fatty acids which are obtained by fractionation from vegetable oils. Carbon substrate cost can significantly contribute to the total production cost and may be as high as 40 % (Choi and Lee 1997). Given that the current commodity price for a metric ton of octanoic acid is $3.34K–4.90K US (http://www.icispricing.com/il_shared/Samples/SubPage227.asp; http://www.alibaba.com/showroom/caprylic-acid-price.html) compared to soybean oil (US $1.12K) or canola oil (US $1.15K) (http://www.ers.usda.gov/data-products.aspx), PHA production cost may be reduced if the growth phase could be done using a carbohydrate substrate, as in this study. The cost of sugar substrates is significantly lower, for example, the 2013 prices for dried high fructose corn syrup, specifically HFCS-42, and dried glucose syrup are US $639 and US $793 per metric ton (http://www.indexmundi.com/commodities/), respectively. This paper clearly demonstrates the feasibility of scl–mcl–PHA production with a carbohydrate substrate like fructose being used for growth and canola oil for the accumulation phase in wild type *C. necator*. However, this bacterium can typically accumulate in excess of 80 % PHA. López-Cuellar et al. (2011) produced biomass containing 90 % scl–mcl PHA. Achieving this percentage of PHA should lead to an economical commercial process providing that the material produced is of commercial value.

Thermomechanical analysis demonstrated that the incorporation of even a very small amount (less that 0.2 mol%) of mcl monomers can reduce melting point and crystallinity as well as substantially decreasing brittleness. However, the amount of mcl monomer should be further increased as the melting temperature was still high enough to cause difficulties (degradation) during melt processing. Inexpensive processes that can accomplish this should be investigated. For example, inhibition of β-oxidation can be used to increase mcl content (Green et al. 2002). Therefore, one approach to produce large quantities of PHA with higher mcl content using wild type *C. necator* would be to inhibit β-oxidation while feeding both an alternative carbon and energy source, such as fructose, during the PHA accumulation phase. This should allow economical production of a more commercially useful material than homopolymeric PHB.

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