Metabolic Engineering *Sinorhizobium meliloti* and *Pseudomonas putida* for Novel Polymer Production

by

Tam Thanh Tran

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Sinorhizobium meliloti belongs to nitrogen-fixing Rhizobia, the members of the Rhizobiales order of the α-proteobacteria. It can establish a symbiotic relationship with the *Leguminosae* family of flowering plants. It is also able to accumulate poly(3-hydroxybutyrate) (P(3HB)) naturally in the presence of excess carbon source. Its genome sequence, has been fully annotated and well-defined. In spite of its important applications in agriculture, its potential applications in industry have not been paid much attention. We aimed to develop this strain as a robust platform for the production of novel bioplastics which can eventually replace persistent fossil-fuel plastics.

Two engineered genes including *pct*, encoding propionate CoA transferase and *phaC*, encoding PhaC synthase, were partially codon-optimized in *S. meliloti*. Subsequently they were introduced into the strains by either being integrated into the chromosome or being placed on an expression plasmid. The integrant strain which has engineered genes expressed under the native promoter is able to produce P(3HB-*co*-LA) up to 15% dry cell weight (DCW) constituting 30% mol lactic acid (LA). When genes were put in the expression plasmid and expressed under the inducible promoter, phenotype complementation was observed in the *phaC* mutant strain. However, only P(3HB) was able to be produced using this system. We also observed the adverse effects of the induction on polymer production. The higher the induction, the more enzyme activity of reporter gene was detected, but the less amount of P(3HB) was produced.

S. meliloti is a native short chain length (SCL) polyhydroxyalkanoate (PHA) producer, while *P. putida* is a native medium chain length (MCL) PHA producer. Unlike *S. meliloti* which has not been well-studied for non-P(3HB) polymer production, *P. putida* has been drawn a great

deal of attention to be engineered for the production of various types of polymer. Therefore, we would like to discover our plasmid-based system in this strain. The results are very promising since it is, to our knowledge, the first time the P(LA-*co*-3HB-*co*-3HHx-*co*-3HO) has been discovered to be produced in bacterial cells.

Promoter engineering is also a critical strategy in metabolic engineering. A range of promoters of different strengths, which have been well-studied, were selected. The results are very interesting because the relative activities were changed in the presence of synthesized genes. The recovery of promoter strengths in the absence of synthesized genes indicated that the promoter strengths depend on genetic context. Analysis of transcript abundance was consistent with the *gusA* reporter gene measurements.

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listen to me and share my happiness as well as my sorrow. I am also thankful to my Step-Mom for taking a good care of my Dad during the time I study abroad.

The most important person in my life is my Mom who passed away several years ago, even before I entered the program. However, she sacrificed her whole life for bringing me up and inspiring me. She gave me the strength to face the failure and move on. I miss and love you tons, Mom!

In Vietnamese for my family part

Cuối cùng nhưng không kém phần quan trọng, con nợ gia đình rất nhiều vì những khuyến khích và hỗ trợ rất quí giá. Ba vốn nghiêm khắc với con nhưng luôn quan tâm, lo lắng và hi sinh rất nhiều cho con. Tình yêu vô bờ bến ấy là nguồn động lực to lớn cho con để vượt qua những khó khăn. Em gái cũng là người bạn thân, luôn lắng nghe và chia sẻ những vui buồn trong cuộc sống. Con cũng cám ơn Má vì đã chăm sóc Ba thay con khi con phải học xa nhà.

Người quan trọng nhất trong cuộc đời con là Mẹ dấu yêu người đã không còn ở với con nữa. Mẹ đã hi sinh cả cuộc đời để nuôi dưỡng và truyền cảm xúc cho con. Mẹ cho con sức mạnh để đối mặt với thất bại và tiếp tục chiến đấu. Con luôn nhớ và yêu Mẹ nhiều lắm, Mẹ ơi!

Claims of Contribution to Scientific Knowledge

- This is the first attempt to engineer *Sinorhizobium meliloti* and *Pseudomonas putida* for novel polymer production by optimizing and introducing two previously engineered genes, *pct* encoding propionate CoA transferase from *Clostridium propionicum* DSM 1682 and *phaC* encoding PhaC synthase from *Pseudomonas sp.* MBEL 6-19
- Chromosome-engineered *S. meliloti* strain designated as SmUW254 was constructed. SmUW254 showed phenotypic complementation described as mucoid and white color. The strain also exhibited fluorescence when growing on YEM media supplemented with mannitol and Nile Red. The result indicates that the chromosome-engineering system is functional in the strain.
- P(3HB-*co*-LA) copolymer was produced in chromosome-engineered strain SmUW254 up to 15% DCW with 30% mol LA. This is the first time *S. meliloti* produces this type of polymer.
- Purification of P(3HB-*co*-LA) copolymer was performed so that the structure of P(3HB-*co*-LA) was able to be confirmed by NMR and GC/MS. The result is consistent with the result from GC-FID.
- Expression vector pTAM which contains these aforementioned genes was constructed from pTH1227. The pTAM plasmid was then introduced into *phaC* mutant *S. meliloti* and was designated as SmUW256. SmUW256 also exhibited fluorescence when growing on the YEM plate supplemented with mannitol and Nile Red in both inducing and non-inducing conditions. The result shows that the plasmid-based engineering system is also functional in the strain.

- Only P(3HB) was able to be produced in SmUW256 around 30% and 15% DCW in non-inducing and inducing conditions, respectively. This shows that the plasmid-based engineering system was not able to incorporate LA monomer to produce novel polymer.
- GusA activity was investigated when inducing SmUW256 with different IPTG concentrations (0, 0.05, 0.4 and 1mM) in a period of 3-day cultivation. The control strain SmUW255 that contains the empty plasmid pTH1227 showed a tightly regulated promoter with very little leaky GusA activity at the background level. Meanwhile, the plasmid-based engineered strain SmUW256 lacked the tight regulation of the *tac* promoter resulting a higher leaky GusA activity at the background level. The highest GusA activity was obtained when inducing SmUW256 at the IPTG concentration of 1 mM; however, this activity was not as high as the activity obtained in the control strain SmUW255 at the same growth condition.
- GusA activity was investigated when inducing SmUW256 at different time points (no induction, 1 day-, 2 day- and 3 day-induction) in a period of 3-day cultivation. Again, the control strain SmUW255 showed a tightly regulated promoter with very little leaky GusA activity at the background level. Meanwhile, the plasmid-based engineered strain SmUW256 lacked the tight regulation of the *tac* promoter resulting a higher leaky GusA activity at the background level. The highest GusA activity was obtained when inducing SmUW256 at the beginning of cultivation; however, this activity was not as high as the activity obtained in the control strain SmUW255 at the same growth condition.
- P(3HB) accumulation was investigated when inducing SmUW256 with different IPTG concentrations (0, 0.05, 0.4 and 1mM) in a period of 3-day cultivation. Unexpectedly, the highest P(3HB) content of 25% DCW was obtained most at the background level.

P(3HB) content of roughly 15% DCW was obtained when the strain was induced with either 0.05 or 0.1 mM IPTG. P(3HB) content of roughly 10% DCW was obtained when the strain was induced with either 0.4 or 1 mM IPTG. This indicates that P(3HB) production was most efficient at the background level.

- P(3HB) accumulation was investigated when inducing SmUW256 with 0.4 mM IPTG at different time points in a period of 3-day cultivation. The P(3HB) content appeared to remain mostly unchanged (25% - 30% DCW) for 1-day, 2-day or no induction. P(3HB) content remarkably dropped to 15% DCW when the strain was induced at the beginning of cultivation.
- A strain that had the whole P(3HB) synthesis pathway blocked and be equipped with the pTAM plasmid produced PLA up to 3% DCW when cultured in YEM supplemented with lactic acid as a sole substrate. This is the first time PLA was produced in *S. meliloti.*
- Multiple mutant *S .meliloti* backgrounds which are deficiency in P(3HB) cycle pathway was unable to produce LA precursors, which was observed in the plasmid-based engineered strain SmUW256.
- The *P. putida phaC* mutant strain harbouring TAM plasmid (PPUW19) showed a milky white phenotype resembling the phenotype of the wild-type harbouring pTAM (PPUW21) when growing on LB plates supplemented with sodium octanoate. By comparison, the wild-type and mutant strains harbouring the empty plasmid pTH1227 had a yellowish colony color. The result indicates that the system was also functional in *P. putida.*
- P(3HB-*co*- LA-*co*-3HHx-*co*-3HO) was first produced in both PPUW19 and PPUW21 when growing in defined media supplemented with either sodium octanoate or lactic

acid. 3HB and LA fraction were obtained more in PPUW19 by comparison with those in PPUW21. The highest dry cell weight (DCW) and polymer content were achieved in PPUW18 up to 1.3 g/L and 40%, respectively. LA fraction was not able to be detected in the wild-type *P.putida* strain harbouring the empty plasmid pTH1227 (PPUW20). This is the first time this type of polymer was found to be produced in bacteria, specifically *P. putida.*

- P(3HB-*co*-LA-*co*-3HHx-*co*-3HO) was also produced in both strains PPUW19 and PPUW21 when growing in LB media supplemented with either sodium octanoate or lactic acid. The strain PPUW20 hardly produced polymers as efficient as strains harbouring pTAM.
- Several native *S. meliloti* promoters of different strength were selected and introduced into pTAM to replace the inducible Tac promoter and drive the expression of the aforementioned synthesized genes and *gusA* reporter gene. It was found out that the relative promoter activities which were indirectly assessed via GusA activity were not consistent with the results in a previous study.
- Removal of the insert brought the activities back to the initial activities that have been reported in previous study, suggesting the sequence in the synthesized genes influenced the promoter strength.
- The abundance of RNA analyzed by dot blot seems correlated to the results given by indirect measurement of GusA activity; therefore, the change might occurred at the transcriptional level.

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Glossary

BSA Bovine serum albumin

DCW Dry Cell Weight

g gram

GC Gas Chromatography

GC/MS Gas Chromatography/Mass Spectrotometry

Gm Gentamycin

hr hour

kDa Kilodalton

Km Kanamycin

l Litre

LB Luria Bertani broth

M Molar

mA Milliamp

MCS Multiple cloning site

mg Milligram

min Minute

ml Millilitre

mM Milimolar

MCL Medium-Chain-Length

Nm Neomycin

NMR Nuclear Magnetic Resonance

PHA Polyhydroxyalkanoate

PE Polyethylene

PP Polypropylene

PS Polystyrene

PET Poly(-ethylene terephthalate-)

PVC Poly(-vinylchloride-)

P(3HB) Poly(3-Hydroxybutyrate)

PLA Poly Lactic Acid

P(3HB-*co*-LA) Poly(3-Hydroxybutyrate-*co*-Lactic Acid)

SDS PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SCL Short-Chain-Length

3HB 3-hydroxybutyrate

3HHx 3-hydroxyhexanoate

3HO 3-hydroxyoctanoate

Chapter 1 Literature review

1.1 Introduction

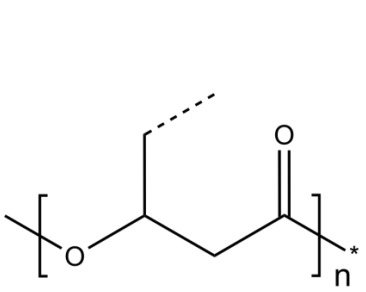
Since the 1940s, petroleum-based plastics have become ever more prevalent. Plastics have been major players in the development of modern society, and they are now one of the most important classes of structural materials. These plastics are made from various synthetic polymers such as polyethylene (PE), polypropylene (PP), polystyrene (PS), poly(-ethylene terephthalate-) (PET) and poly(-vinylchloride-) (PVC) (Amass et al. 1998). There are, however, some inevitable drawbacks. Chief among these, the use of nonrenewable sources of material for the production of commodity plastics as well as accumulation of waste in landfills and the oceans poses a direct threat to wildlife and human health. Hence, it is ultimately necessary to substitute new biodegradable materials for these traditional plastics.

A variety of potential biodegradable plastics have been intensively and extensively investigated, such as polyhydroxylalkanoates (PHA), polylactic acid (PLA), polyglycolic acid (PGA), and blends of these polymers (Amass et al., 1998; Kolybaba et al., 2003). These polymers are produced in microorganisms from renewable source, or from renewable source using synthetic methods. Biodegradable polyesters are able to be commercially produced through chemical synthesis, using either polycondensation or ring-opening polymerization. However, polymer production based on chemical processing still encounters a number of serious issues such as requirement for high temperature, long reaction times, and uncontrollable chain length or monomer restriction (Jung et al., 2010b; Okada, 2002). Although major rationales for producing these polymers are environmental preservation and human health protection, this is not always achieved. For example, chemical synthesis of PLA results in harmful left-over chemical residues

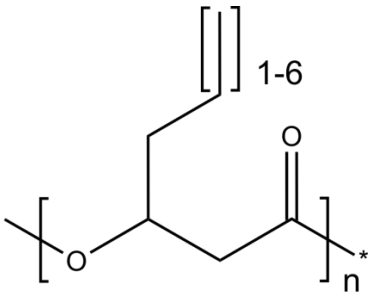
from the metal catalysts used in the reaction (Taguchi et al., 2008). The recent trend of using enzymes as catalysts is more advantageous than organic synthesis due to higher regiospecificity and stereospecificity. However, problems with maintenance of catalytic enzyme activity in non-aqueous media and the expensive cost of enzyme purification hinder the production of these polymers on the industrial scale (Gross et al., 2001; Okada, 2002).

1.2 Types of PHAs

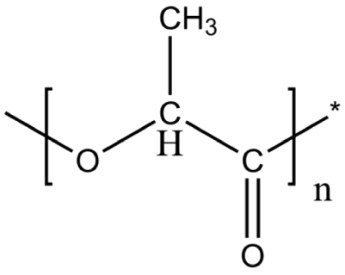
PHA is a native product accumulated in various types of bacteria as carbon and energy storage materials under nutrient-limiting conditions in the presence of an abundance of carbon source (Anderson and Dawes, 1990). A variety of bacteria have been reported as native PHA producers such as *Cupriavidus necator* H16 (formerly, *Ralstonia eutropha* H16) (Doi et al., 1992; Peoples and Sinskey, 1989; Schubert et al., 1988; Slater et al., 1988), *Alcaligenes latus* (Hrabak, 1992; Braunegg and Bogensberger, 1986), *Pseudomonas* (Fritzsche et al., 1990; Gross et al., 1989; Huisman et al., 1991; Timm et al., 1994; Timm and Steinbuchel, 1992), *Aeromonas* (Doi et al., 1995), *Rhodococcus ruber* (Pieper and Steinbiichel, 1992). *Syntrophomonas wolfei* (McInerney et al., 1992) and the rhizobia (Trainer and Charles, 2006). PHAs consist of various monomer units in D(-) configuration ranging from 3 to 16 carbon monomers. This wide range of monomers provides PHAs with diverse material properties, thermoplastics classified as short chain length (SCL) PHAs consisting of 3- to 5-carbon monomers, elastomers as medium chain length (MCL) PHAs consisting of 6- to 16-carbon monomers and their copolymers showing intermediate properties. While over 100 types of PHAs have been described, the most common is the SCL-PHA, poly-3-hydroxybutyrate (P(3HB)).



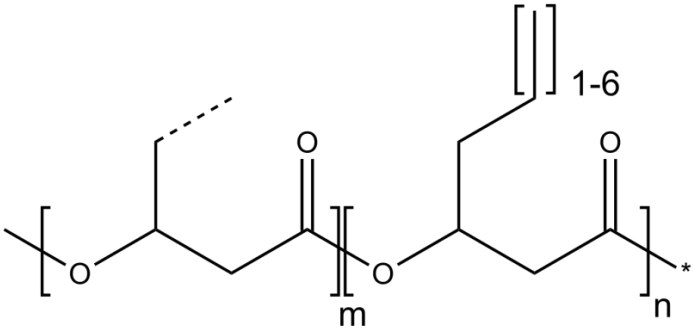
SCL PHA



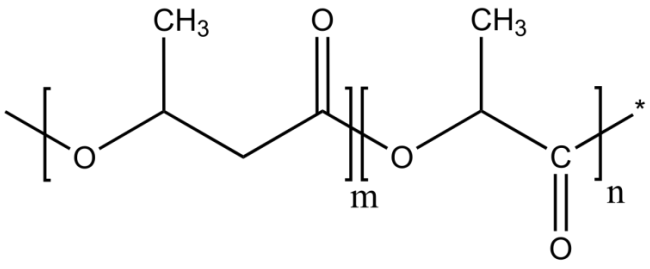
MCL PHA



PLA



SCL-MCL PHA



P(3HB-*co*-LA)

Figure 1.1 Chemical structure of different types of polymers

1.3 PLA/ P(LA-*co*-3HB) copolymer

Polylactic acid (PLA) is a biodegradable polymer that is currently produced chemically from biologically produced lactic acid derivatives using heavy metal catalysis (Taguchi et al., 2008), but is not known to be naturally produced by any microbe. It possesses several favourable properties such as high strength, biocompatibility, heat resistance, permeability and optical activity that make it a leading candidate for medical applications such as resorbable sutures, drug carriers, organ-substituting implants and artificial blood vessels (Lunt, 1998; Södergård and Stolt, 2002). The co-polymer PLA-*co*-PHA has been obtained through ring-opening polymerization of L-lactide combined with PHA using stannous octoate as a catalyst as shown in Figure 1.2 (Haynes et al., 2007). In part to avoid contamination with trace levels of heavy metal (Figure 1.3), research has focused on producing PLA and similar types of polymers through direct fermentation (Selmer et al., 2002; Jung et al., 2010; Yang et al., 2010), despite the absence of natural production. It was determined that lactic acid (LA) and 3-hydroxyalkanoate (3HA) monomer, especially D-3-hydroxybutyrate, share a similar structure including both hydroxyl and carboxyl groups essential for the formation of an ester bond (Yang et al., 2010).

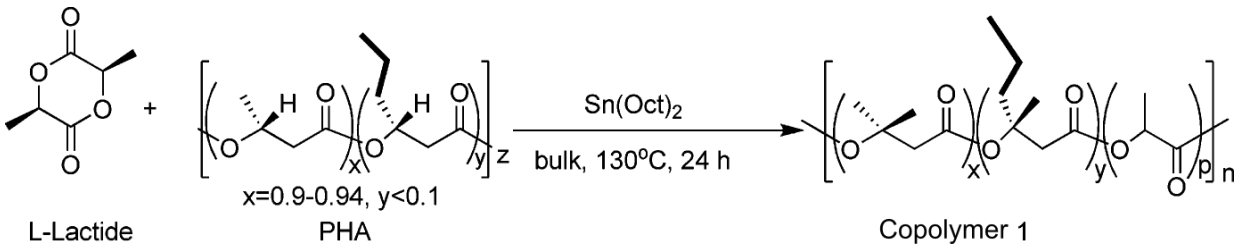


Figure 1.2 Synthesis of PHA-co-PLA via ring-opening polymerization (Adapted from Haynes et al., 2007)

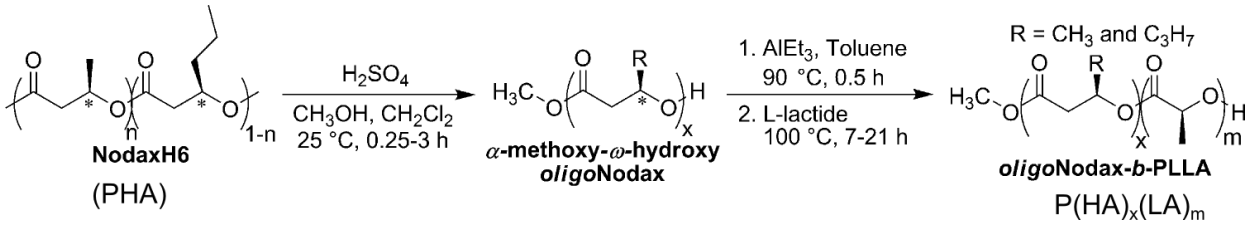


Figure 1.3 Synthesis of oligoNodax-b-PLLA (Adapted from Schreck and Hillmyer, 2007)

1.4 Physical properties of different types of polymers

Physical characterization of PLA, PHA and novel copolymers has been carried out in previous studies to enhance favourable properties and circumvent individual drawbacks of each homopolymer (Haynes et al., 2007; Noda et al., 2004; Noda et al., 2005; Schreck and Hillmyer, 2007). The properties of the polymer can be well-defined based on the type, content, monomer composition, as well as molecular weight and molecular weight distribution. P(3HB) homopolymer exhibits undesirably high crystallinity which causes a limited range of applications due to its hard and brittle properties (Noda et al., 2005). Another disadvantage of P(3HB) is that it is difficult to process using the melting method since it has a high melting temperature (>170ºC) which is close the thermal decomposition temperature. Incorporation of another SCL 3HA (3HV) did not help to circumvent these limitations. Meanwhile, MCL PHAs which contain higher carbon number (6-14 carbons) are described as having low melting points, high elasticity and biodegradability (Jiang et al., 2006). Since the melting temperature of MCL PHAs is pretty low varying from 39ºC to 61ºC,materials made from MCL PHAs are not rigid enough and tend to lose their coherence at rather low temperature (Koning, 1995). However, incorporation of a small amount of MCL PHAs into P(3HB) showed some improved properties of the target polymers. Their properties were described as similar to polyethylene, which is relatively tough and ductile. The crystallinity and melting temperature (Tm) of P(SCL-*co*-MCL 3HA) are decreased significantly in comparison with the traditional PHA polymers, such as P(3HB) or PHBV copolymers. Therefore, this type of copolymer was able to be processed efficiently and in a cost-saving way. Another advantage is that the complete degradation of these copolymers can be achieved in either aerobic or anaerobic environments.

It was reported that PLA has a glass transition temperature (Tg) of ca. 60ºC and Tm of ca. 170ºC, whilst Tg of PHA varies from -10ºC and 0ºC, and its Tm fluctuates between 160ºC and 180ºC (Haynes et al., 2007). Physicochemical properties of P(3HB-*co*-LA) including the molecular weight, thermal properties, and melt viscosity were taken into account as well. The mole fraction of LA monomer was inversely proportional to the molecular weight and the crystallinity of P(3HB-*co*-LA), and directly proportional to the glass transition temperature (Tg) of the polymer (Yang et al., 2010). While Tg was apparently affected by LA fraction, melting temperature (Tm) was just slightly changed according to LA monomer composition and maintained around 160ºC. Copolymer showed a decreased molecular weight of 29,000 compared to that of PHA which had a molecular weight of 126,000. Also, this copolymer exhibits more favourable changes in thermal behavior as well as the glass transition and the crystallization transition. Furthermore, improved mechanical properties, such as lower viscosity and dynamic moduli which PLA alone cannot possess, have been obtained for this copolymer. There was also an attempt to blend MCL PHA with PLA using a melt-mixing method and the properties of the new blended polymers were examined (Noda et al., 2004). It was reported that the new polymer possessed much improved properties such as increased toughness and ductility as well as optical clarity.

1.5 *Sinorhizobium meliloti* and its P(3HB) pathway

Sinorhizobium meliloti, a soil bacterium, best known for its N_2-fixing symbiosis with *Medicago sativa* (alfalfa), is genetically amenable and metabolically versatile (Bélanger et al., 2009b; Gurich and González, 2009). It has the advantage of having been used for many years in the agricultural inoculant industry, and is culturable to very high titres. It is a native P(3HB) producer that can accumulate P(3HB) on the order of 60-80% cell dry weight under growth-

limiting conditions in the presence of excess carbon source (Trainer and Charles, 2006). The P(3HB) cycle in *S. meliloti*, which includes the well-studied P(3HB) synthesis and degradation pathways (Figure 1.4), resembles the P(3HB) cycles in other native P(3HB) producers (Charles et al., 1997). The first step in this cycle is the conversion of acetyl-CoA into acetoacetyl-CoA catalyzed by *phbA*-encoded ketothiolase (EC 2.3.1.9). This is followed by acetoacetyl-CoA reductase (EC 1.1.1.36) encoded by *phbB* to convert acetoacetyl-CoA into 3-hydroxylbutyryl-CoA. In the last step of the P(3HB) synthesis pathway, *phbC*-encoded P(3HB) synthase (EC 2.3.1.B2) is responsible for polymerizing 3-hydroxylbutyryl-CoA into PHB. In *S. meliloti*, other enzymes taking part in the P(3HB) degradation pathway are P(3HB) depolymerase (*phaZ*) (EC 3.1.1.75), D-3-hydroxybutyrate dehydrogenase (*bdhA*) (EC 1.1.1.30), and acetoacetyl-CoA synthetase (*acsA2*) (EC 6.2.1.16). The P(3HB) depolymerase carries out the first step to cleave P(3HB) into D-3-hydroxybutyrate monomers. Next, these monomers are oxidized and converted to acetoacetate by the action of D-3-hydroxybutyrate dehydrogenase. The last step which supplies P(3HB) production precursors (acetoacetyl-CoA) is catalyzed by acetoacetyl-CoA synthetase.

Interestingly, the P(3HB) synthase-encoding gene, is not co-localized with other P(3HB) synthesis genes in the genome of *S. meliloti* (Trainer and Charles, 2006). This pattern was found to be common among other PHB-accumulating bacteria such as *Caulobacter crescentus*, *Azorhizobium caulinodans*, *Rhizobium etli*, *Paracoccus denitrificans* and *Methylobacterium extorquens*. Meanwhile, in the β-proteobacterium *C. necator* for example, three P(3HB) biosynthesis genes are located in a single operon in the order of *phaC*, *phaA* and *phaB*. These genes are arranged in the opposite order in the γ-proteobacterium *A. vinelandii*. Although *phbC*

is located by itself in the *S. meliloti* genome, the two other genes (*phbA* and *phbB*) are found within a separate operon.

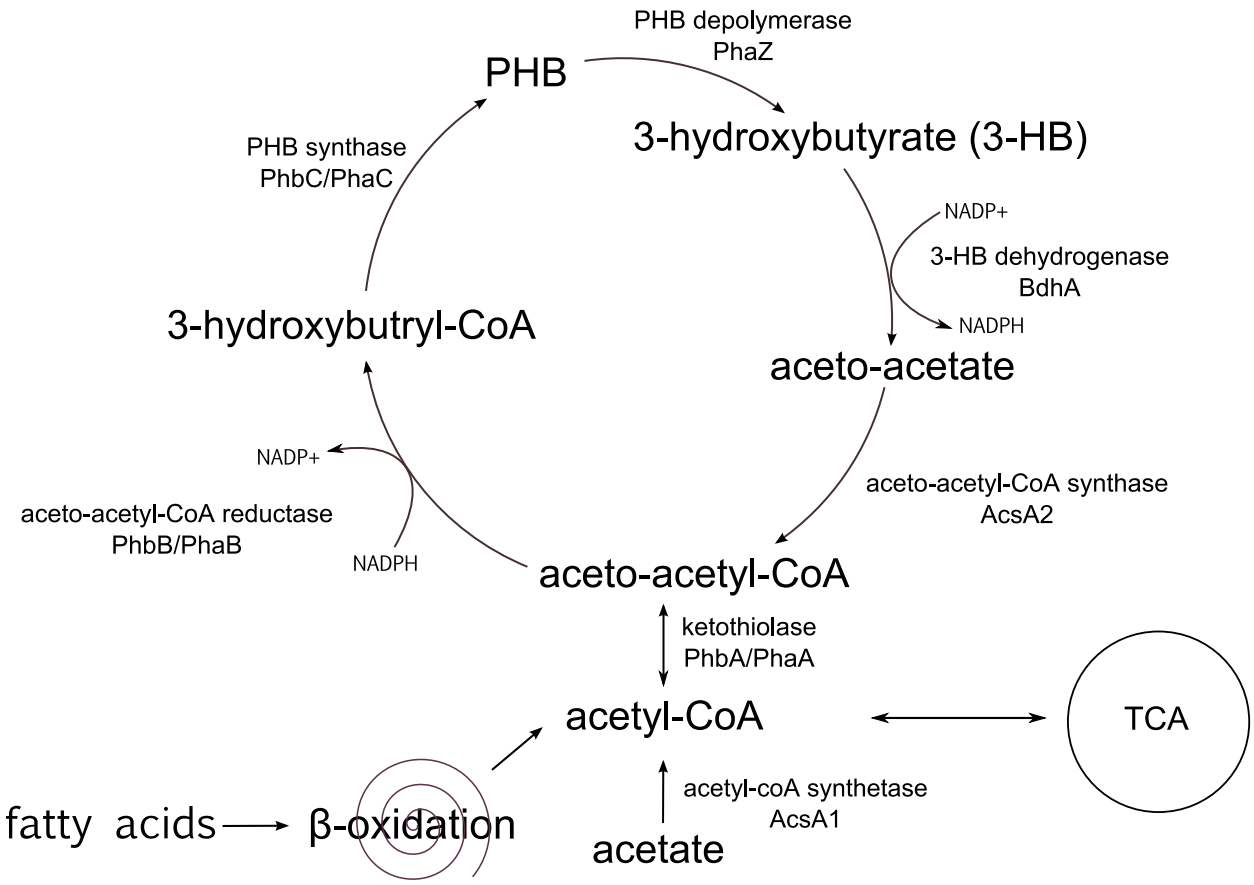


Figure 1.4 P(3HB) biosynthesis and degradation pathways

1.6 *Pseudomonas putida* strain and MCL-PHA pathway

Pseudomonas putida KT2440 and its rifampicin-resistant derivative KT2442 have been employed extensively to investigate the capability of MCL PHA production. These laboratory workhorses are unable to accumulate PHB, but able to polymerize MCL PHA during unbalanced growth conditions up to 75% PHA (Sun et al., 2007). This characteristic was also found among other *Pseudomonas* strains belonging to rRNA homology group I such as *P. aeruginosa* and *P. oleovorans* (Huisman et al., 1989; Huisman et al., 1992). Normally, the majority of monomers produced in these strains have the same number of carbons as the substrates provided to the cultures; others could be cleaved by one or more C_2 units. Depending on the type of growth substrates provided, two main metabolic pathways operate in these strains to supply precursors for MCL PHA production, the β-oxidation pathway and the fatty acid *de novo* biosynthesis pathway. Feeding the strains with medium- or long-chain alkanols/ fatty acids could lead to accumulation of MCL PHA through the β–oxidation pathway, while using glucose and other substrates that are unrelated to the above mentioned substrates can supply MCL PHA precursors through the fatty acid *de novo* biosynthesis pathway (Figure 1.5) (Huijberts et al., 1992). These pathways are the sources to supply substrates of the PHA substrates, namely 3-hydroxyacyl coenzyme A thioesters. In addition to these two main pathways, there is another reported side metabolic route of supplying 3-hydroxyacyl-CoA from acetyl-CoA through chain elongation reaction when hexanoate is used as a substrate (Hoffmann and Rehm, 2004a; Huijberts et al., 1994). PHA synthesis was found to start in exponential growth phase, reach the maximum amount of 50% cell dry weight at the beginning of stationary phase, and then remain unchanged thereafter (Huisman et al., 1992).

PHA biosynthesis genes in *P. putida* are located in the *phaC1ZC2D* operon which is composed of two PHA polymerase genes (*phaC1* and *phaC2*), a depolymerase gene (*phaZ*), and a transcriptional regulatory gene (*phaD*) (Luengo et al., 2003). An additional cluster of two phasin genes (*phaI* and *phaF*) involved in PHA biosynthesis is located downstream of *phaC1ZC2D*, arranged in the opposite orientation. The *phaF* gene is known to be a negative regulator which represses the transcription of the PHA synthase gene (*phaC1*) and the other phasin gene (*phaI*) (Hoffmann and Rehm, 2004b). The more PHA is accumulated in the strain, the more phasin protein PhaF is bound to the surface of the PHA granule; hence, less freely available PhaF could lead to the transcriptional enhancement of *phaC1* and *phaI*. The key enzyme, PhaG transacylase, plays an intermediate role by linking fatty acid *de novo* biosynthesis with PHA biosynthesis by replacing the acyl carrier protein moieties with CoA thioesters (Rehm et al., 1998; Hoffmann et al., 2002). Owing to the PhaG enzyme, the strain is able to produce PHAs from non-related carbon sources such as sugars and gluconate.

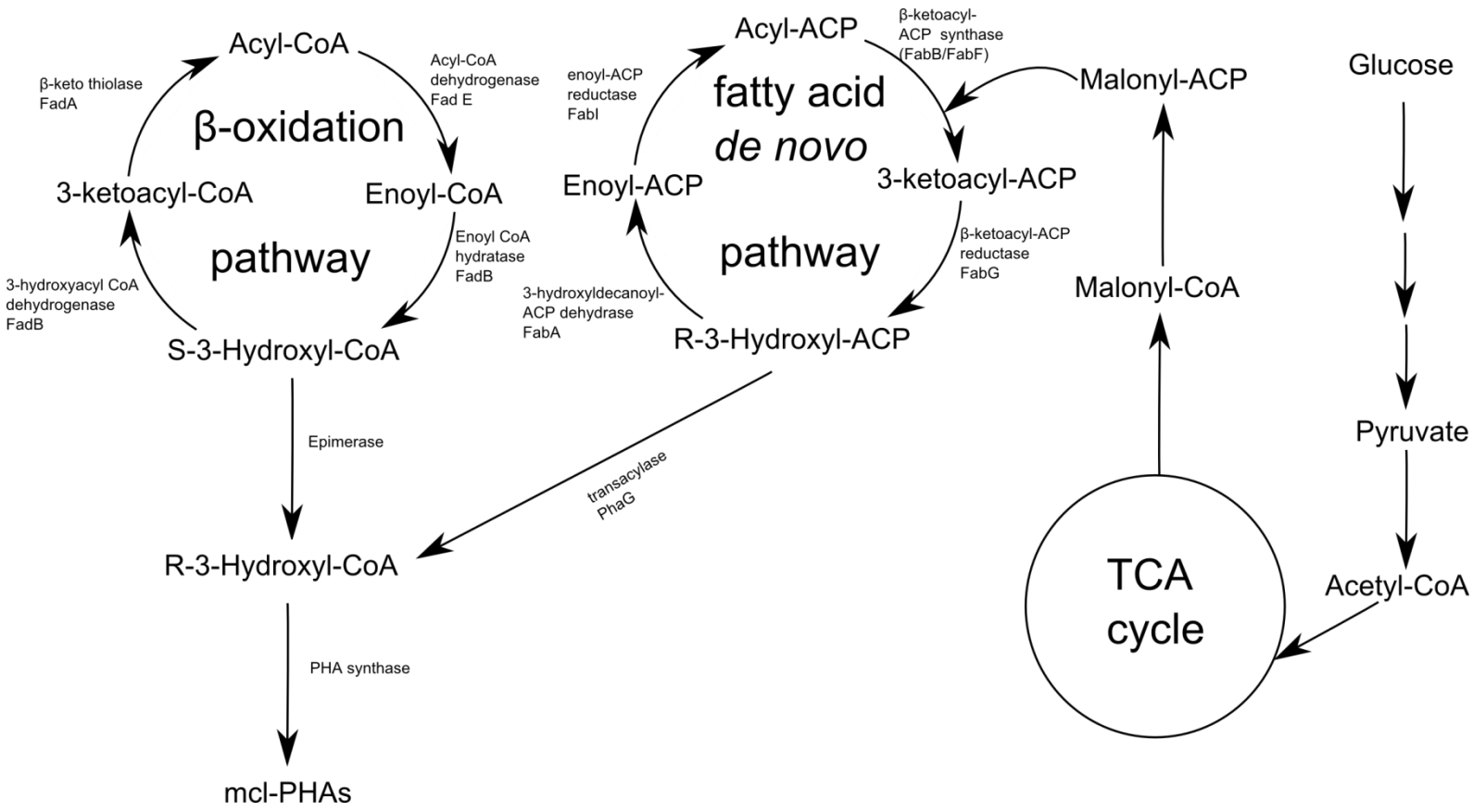


Figure 1.5 MCL PHA production pathway in *P. putida*.

1.7 Metabolic engineering in native producers

There are a number of different strains which naturally accumulate polymers under certain conditions such as nutrient limitation (N, P, Mg, or O) or carbon source abundance (Anderson and Dawes, 1990). These strains includes *Cupriavidus necator* H16 (formerly, *Ralstonia eutropha* H16), *Alcaligenes latus*, *Pseudomonas putida* KT2440, *Aeromonas*, *Rhodococcus ruber*, *Syntrophomonas wolfei* and others. However, these native polymer producers still need to be improved to further meet the industry demands for commercialization in terms of economical profit and intrinsic properties for practical applications. Therefore, these strains need to be further engineered using metabolic tools so that they can meet the industrial demands.

1.7.1 Short-chain-length native producers

Among the short-chain-length native producers, the β-Proteobacteria *A. latus* and *C. necator* are the most popular model systems for in-depth studies in polymer biosynthesis. *C. necator* strain H16 was able to accumulate P(3HB) up to 80% CDW from glucose, exhibited rapid growth, and has the ability to use different substrates including ethanol, glucose and hydrogen/carbon dioxide/air (Byrom, 1987; Holmes, 1985). P(3HB) produced by *C. necator* was successfully commercialized by a company called ICI. However, due to its brittle properties, there are challenges to find alternative materials with better properties, yet their production remains equally cost-effective. Surprisingly, in addition to the primary 3HB monomer, a small amount of MCL PHAs (3HHx and 3HO) were detected in fatty acid β-oxidation inhibited *C. necator*. In this study, sodium acrylate was used as a *β*-oxidation inhibitor and sodium octanoate as a carbon source (Green et al., 2002). Applying two-stage cultivation resulted in the increase of 3HHx (10.2%) and 3HO (1.6%), and 3HP (6.5%). Substitution of the native *C. necator* PHA

synthase with the *P. aeruginosa* Class II PHA synthase PhaC1 also led to MCL production (3HHx, 3HO and 3HD) in the presence of various acrylic acid concentrations and with decanoate as the carbon source. Copolymer P(3HB-*co*-1.5% mol 3HHx) up to 48% DCW was achieved in recombinant *C. necator* strains harbouring crotonyl-CoA reductase gene from *Streptomyces cinnamonensis* (ccr_{Sc}) and PHA synthase and (*R*)-specific enoyl-CoA hydratase genes from *Aeromonas caviae* ($phaC\text{-}J_{Ac}$) using fructose as a substrate (Fukui et al., 2002). After further development, the same copolymer P(3HB-*co*-3HHx) was also obtained with very high polymer content (40% - 87% DCW) and 3HHx fraction up to 5% mol from renewable substrates (palm kernel oil, palm olein, crude palm oil and palm acid oil) in engineered *C. necator* strains harbouring the PHA synthase gene from *Aeromonas caviae* (Loo et al., 2005). Expressing tailor-made derivatives of PHA synthase from *Aeromona caviae* in *C. necator* enabled production of P(3HB-*co*-3HHx) with adjustable 3HHx monomer composition (Tsuge et al., 2004). These mutated PHA synthases were made to increase enzyme activity towards the (R)-3HB-CoA monomers by using *in vitro* evolutionary engineering techniques.

1.7.2 Medium-chain-length native producers

Pseudomonas strains were considered as a model system for further development of MCL-PHA production because of their ability to produce MCL PHAs naturally. To allow production of SCL/MCL PHA copolymer, specifically P(3HB-*co*-3HHx), a 5-kb fragment from *Aeromonas caviae* genomic DNA which included the PHA synthase gene ($phaC_{Ac}$) together with four open reading frames (ORFs) and one putative promoter region was introduced into *P. putida* PHA-negative mutant (Fukui and Doi, 1997). It was revealed that ORF3 showed (R)-enoyl-CoA hydratase activity, hence it supplied (R)-3-hydroxyacyl-CoA monomer units. Since it was known that the transacylase gene (*phaG*) links the fatty acid *de novo* synthesis and PHA synthesis, two

different *phaG* genes from *P. putida* and *P. oleovorans* were examined in different *Pseudomonas* strain backgrounds (Hoffmann et al., 2000). Both genes functioned similarly in a *phaG* mutant of *P. putida* KT2440 which produced MCL PHAs of similar monomer composition. More interestingly, *P. oleovorans*, which marginally accumulated MCL PHA from non-related carbon sources even under inducing conditions was then recovered to produce MCL PHA by introduction and expression of *phaG* from either *P. putdia* or *P. oleovorans*. A defective β-oxidation pathway in *P. putida* resulted in MCL PHA production with a high content of a particular monomer (Liu and Chen, 2007). The *fadA fadB* mutant of *P. putida* was able to accumulate MCL PHAs consisting of 31- 49 mol% 3-hydroxytetradecanoate as a major monomer when using tetradecanoic acid as a sole carbon source. The same mutant strain accumulated 50% DCW MCL PHAs higher than the parent strain KT2440 when dodecanoate was used as a carbon source (Ouyang et al., 2007). In addition, the monomer composition obtained in the mutant strain had 3-hydroxydodecanoate content that was 5 times greater than that of the parent strain. This 3HDD fraction even further increased up to 75% in a two-step fermentation process after 16 h cultivation, and then decreased over time. For an efficient PHA production, PHA concentration and PHA productivity need to be taken into account. It was found out that an optimized initial KH_2PO_4 concentration increased PHA concentration and PHA content up to 72.6 g/l and 51.4% CDW, respectively, in *P. putida* by fed-batch cultivation using oleic acid as a substrate (Lee et al., 2000). As mentioned earlier, substrate cost contributes a major portion of PHA production cost. Xylose, a hemicellulose derivative, is one of the most inexpensive carbon sources. MCL PHAs could be obtained in engineered *P. putida* KT2440 from xylose and octanoic acid when two genes encoding xylose isomerase (XylA) and xylulokinase (XylB) from *E. coli* W3110 were expressed in the *P. putida* strain (Le Meur et al.,

2012). Technically, xylose was used to support the cell growth, while octanoic acid was to supply MCL PHA precursor. The combination of non-related and related carbon source is a feeding strategy to avoid unnecessary use of expensive fatty acids. Bioinformatics also provides powerful tools to construct a robust *Pseudomonas* system by identifying target genes to be either deleted or over-expressed. For example, based on a genome-wide *in silico* prediction, genes encoding for pyruvate dehydrogenase subunit (AcoA) and glucose dehydrogenase (Gcd) were potential target genes to be engineered for the improvement of MCL PHA production using glucose as sole carbon source (Borrero-de Acuña et al., 2014). Overproduction of AcoA resulted in the increase of MCL PHA by 33% in the wild-type strain, and by 121% in the *gcd* mutant strain.

1.8 Metabolic engineering in non-native producers

1.8.1 In Bacteria: *Escherichia coli*

Escherichia coli has been the most widely used model system in the metabolic engineering field for the reason that it can grow fast in a relatively inexpensive medium, and is easy to lyse. Since it is not a native producer, however, it does not possess a depolymerase that can degrade polymer. Also, there are various options of genetic and molecular tools which have been developed for this specific strain over several decades. These advantages result in a shorter cycle time for the production process, a lower cost of fermenter operation as well as purification, and relatively easy genetic modification.

1.8.1.1 Short chain length (SCL) PHAs, medium chain length (MCL) PHAs and copolymer SCL/MCL PHAs

P(3HB) production in *E. coli* was first investigated almost two decades ago (Lee and Chang, 1995; Choi et al., 1998; Genser et al., 1998). Among different *E. coli* strains, it was found that XL1-Blue was the most promising host for P(3HB) production because it can accumulate up to 81% with the PHA concentration of 7.2 g/l (Lee and Chang, 1995). In a follow-up study, the strain was equipped with either the *A. latus* PHA biosynthesis system ($phaCAB_{Al}$) or *C. necator* H16 PHA biosynthesis system ($phaCAB_{Cn}$). It was reported that the recombinant *E. coli* strain harbouring $phaCAB_{Al}$ showed a higher productivity than the recombinant $phaCAB_{Cn}$ strain (Choi et al., 1998). A similar result was also obtained when using a different *E. coli* strain (Genser et al., 1998). Even though the author pointed out the advantages of using *E. coli* as a P(3HB) production machine and obtained better results when combining this host with the better PHA biosynthesis system $phaCAB_{Al}$, there were still questions about whether such recombinant strains of *E. coli* could outperform the wild-type native PHA producers.

Further studies demonstrated that the gene order of the *phaCAB* operon can contribute to the improvement of the properties of produced P(3HB). By rearranging the order of these genes from *C. necator* and expressing them in *E. coli*, it was found that gene order affects both the molecular weight and level of P(3HB) accumulation. In general, increase of molecular weight was accompanied by decrease in polymer yield. The *phaBCA* gene order represented the best compromise between them. It was also found that the *de novo* fatty acid biosynthesis pathway was able to supply 3HB precursors as long as the strain was equipped with genes encoding certain key enzymes: 3-ketoacyl-ACP synthase III (FabH) or malonyl-CoA-ACP transacylase (FabD) that links this pathway with the PHA biosynthesis pathway. Co-expression of either *fabH* or *fabD* with *phaC* resulted in P(3HB) production of 5-11% CDW (Taguchi et al., 1999).

In addition to P(3HB) homopolymer production in *E. coli*, other studies also attempted to incorporate 3HV, another type of SCL PHA, into P(3HB) to create P(3HB-*co*-3HV) copolymer (Choi and Lee, 1999; Slater et al., 1992; Wong et al., 2008; Yim et al., 1996). The engineering strategy was similar to that used to produce P(3HB) homopolymer except for substrate feeding. In order for *E. coli* strains to be able to produce 3HV monomer, propionate was added into the media to supply sources for 3HV production. A recombinant *E. coli* strain LS5218 harbouring P(3HB) biosynthesis genes from *C. necator* was able to accumulate P(3HB-*co*-3HV) up to 30% mol 3HV (Slater et al., 1992). Varying either glucose or propionate concentration in the media resulted in various 3HV monomer compositions. In another study that investigated the P(3HB-*co*-3HV) accumulation ability among different *E. coli* host strains, XL1-Blue was shown again to be the best *E. coli* strain for this copolymer production in terms of both PHA content and 3HV fraction (Yim et al., 1996). There was no clear explanation for why this strain was able to outperform other *E. coli* strains at P(3HB) production. Unlike other non-*E.coli* strains, this

recombinant strains did not produce copolymer from valerate because Ato enzymes which were required to convert valerate into valeryl-CoA were not induced by valerate. Ato enzymes are the products of the following genes: *atoA* (CoA transferase), *atoB* (thiolase) and a regulatory gene *atoC* (Pauli and Overath, 1972). These enzymes take part in the degradation of acetoacetate to acetate and acetyl-CoA. It was also hypothesized that propionate was transported and activated to propionyl-CoA by an undefined acetate transport system which could include glyoxylate shunt enzymes and acetate-activating systems involving acetate kinase and phosphotransacetylase. Conversion of propionyl-CoA from propionate was also expected to be improved by introducing propionyl-CoA synthetase gene (*prpE*) from *Salmonela enterica* (Wong et al., 2008). However, 3HV was not efficiently incorporated into polymer; hence 3HV molar content was obtained only between 5% and 18% depending on the expression level of PrpE under the control of the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *tac* promoter despite the fact that there was an abundance of propionyl-CoA produced. The reason might be that the PHA operon and conditions were not optimized for higher 3HB proportion.

Since MCL PHAs (C_5-C_{14}) and SCL/MCL copolymer PHAs showed more favourable properties than SCL PHAs, other studies shifted toward engineering *E. coli* for the production of these PHAs. Two strategies which have been used to engineer *E. coli* for MCL PHA production are to engineer either the fatty acid *β*-oxidation pathway or the *de novo* fatty acid biosynthesis pathway. For the fatty acid *β*-oxidation pathway, the approach involved inhibition/ deletion/substitution of the β-oxidation multienzyme complex, such as FadA/FadB (Langenbach et al., 1997; Snell et al., 2002), or overexpression of desired enzymes, such as enoyl-CoA hydratase and 3-ketoacyl-CoA (ACP) reductase (Taguchi et al., 2001), or a combination of these (Park et al., 2002) to increase the precursor flow toward MCL PHA polymerization.

The first study in this line of research was published in 1997 by Langenbach *et al.* (Langenbach et al., 1997). In this study, they investigated the production of MCL PHA in recombinant *E. coli* by expressing the *Pseudomonas aeruginosa* PHA synthase gene *phaC1*. Various *E. coli* strains as well as different media were tested for MCL PHA production. As a result, the *E. coli fadB* mutant strain LS1298 harbouring the above PHA synthase gene cultivated in LB medium containing 0.5% (w/v) decanoate most efficiently produced MCL PHA (21% CDW) consisting of 2.5% mol 3-hydroxyhexanoate, 20% mol 3-hydroxyoctanoate, 72.5% mol 3-hydroxydecanoate and 5% mol dodecanoate (Langenbach et al., 1997). The same group also looked at the polymer production in *E. coli* when expressing the second PHA synthase gene *phaC2* from *Pseudomonas aeruginosa* (Qi et al., 1997). When dodecanoate was used as a carbon source, recombinant *E. coli* produced MCL PHA (15% CDW) composed of 35% mol 3-hydroxydodecanoate, 60% mol 3-hydroxydecanoate and 5 % mol 3-hydroxyoctanoate. Also, the study concluded that the two PHA synthases from *P. aeruginosa* possess similar characteristics which led to similar PHA production in terms of monomer composition and molecular mass.

The expression of the enoyl-CoA hydratase encoding gene *phaJ* from *Aeromonas caviae* was investigated in a recombinant *E. coli* strain (Fukui et al., 1999). The co-expression of this gene and $phaC_{ac}$ resulted in SCL/MCL PHA accumulation (7-11% CDW) in *E. coli* strain LS5218. The homologous *phaJ* genes ($phaJ1_{Pa}$ and $phaJ2_{Pa}$) from *P. aeruginosa*, were also co-expressed with PHA synthase genes from *Aeromonas caviae* ($phaC_{ac}$) or *Pseudomonas sp. 61–3* ($phaC_{Ps}$) in *E.coli* LS5218 [*fadR601, atoC2*(Con)] (Taguchi et al., 2001; Tsuge et al., 2000). Depending on the PHA synthase genes used, different types of SCL/MCL copolymers were produced. The PHA synthase gene $phaC_{ac}$ incorporated more SCL PHA (3HB) into the polymer while the PHA synthase gene $phaC_{Ps}$ favoured MCL PHA incorporation. These results

suggested that these hydratase genes are essential for driving precursors from the *β*-oxidation pathway toward polymer production in *E.coli*.

Following up on this study, other groups searched for enzymes homologous to PhaJ. The role of the *E. coli* enzyme YfcX, which belongs to the crotonase superfamily of enzymes, has been investigated for its association with MCL PHA production in *E. coli* (Snell et al., 2002). The *fadB yfcX* mutant *E. coli* harbouring *P. oleovorans phaC1* was unable to accumulate any MCL PHAs. However, genetic complementation of *yfcX* in this strain restored the ability to produce MCL PHAs from both palmitate and decanoate substrates. Another enzyme in the crotonase superfamily of enzymes, MaoC, has been investigated for homologous function of enoyl-CoA hydratase (Park and Lee, 2003). A *fadB maoC* mutant strain exhibited significantly reduced amounts of MCL PHA accumulated (43%) compared to the *fadB* mutant strain. Restoring the function of the *maoC* gene in the double mutant strain improved the amount of MCL PHA. However over expression of *maoC* in a *fadB* mutant did not result in as much MCL PHA accumulation as was achieved after complementation of FadB in this strain. Also, deletion of both MaoC and FadB impaired MCL PHA accumulation less than deletion of both YfcX and FadB. These results showed that MaoC played a less important role in the β-oxidation pathway than YfcX, hence affected the ability of MCL PHA accumulation less. Along with YfcX and MaoC, five other enzymes including PaaG, PaaF, BhbD, SceH, and YdbU, also have been investigated (Park and Lee, 2004). The wild-type *E. coli* strain W3110 which has a full β-oxidation pathway was unable to produce any MCL PHAs regardless of introducing the PHA synthase gene alone or along with each of the aforementioned genes. However, co-expression of the PHA synthase gene and one of these genes in a *fadB* mutant strain increased the amount of

MCL PHAs accumulated compared to expression of PHA synthase gene alone, and it even increased MCL PHA concentration roughly twice for the expression of *paaG, paaF* and *ydbU.*

The combination of both expression of 3-ketoacyl-ACP reductase genes and deletion of *fadA* could lead to specific monomer enrichment in MCL PHAs (Park et al., 2002). Overexpression of the *E. coli* $fabG_{Ec}$ gene led to MCL PHA rich in 3-hydroxydecanoate monomer up to 93 mol% in *fadA* mutant *E. coli* strain. Other enzymes, such as encoded by *fadD, fadL* and *fadE*, also have been investigated for modulating monomer composition in *fadA* and/or *fadB* mutant *E. coli* strains (Park et al., 2003). Enrichment of 3HD was able to be obtained up to 78% mol by expression of *fadD* gene only in a *fadA* mutant strain. The co-expression of *fadE* and *fadD* resulted in the increase of C10 and C12 monomers when decanoate and dodecanoate were used as the substrates, respectively.

The drawback of targeting the β-oxidation pathway to develop MCL PHA producers is that this involves supplementation with fatty acids, and fatty acids are toxic to the cells and not economically viable. Therefore, sugars were considered to be a more favourable substrate for PHA production. The use of sugars involves engineering an alternative pathway that is the *de novo* fatty acid biosynthesis pathway. The *phaG* gene plays an important role in linking this pathway and the PHA biosynthesis pathway (Park et al., 2005b). Since *E. coli* is not a native PHA producer, the fatty acid biosynthesis pathway does not efficiently supply the source of R-3HA-ACPs which are substrates for the transacylase PhaG. Therefore, expression of the *phaG* and *phaC* genes did not enable this strain to accumulate MCL PHA (Rehm and Mitsky, 2001). However, either the disruption of FabA/ FabI or the chemical inhibition of FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II) did not change this incapacity. Interestingly, the application of triclosan, a specific inhibitor of the enoyl-ACP reductase FabI,

led to a small amount of MCL PHA detected (2-3% CDW) from non-related carbon source when both *phaG* and *phaC* were expressed in *E. coli*. It is still unclear what made the difference between the *fabI* mutant strain and the chemical inhibited *fabI* strain, which allowed only the latter to produce MCL PHA. Triclosan was added to the medium after a 24 hr incubation, so it might be that FabI was required at the beginning to produce MCL PHA precursors.

Another study also proposed alternative transacylase enzymes (FabH, FabD) which also can link the *de novo* fatty acid biosynthesis pathway and the PHA biosynthesis pathway (Taguchi et al., 1999). However, as mentioned earlier, the co-expression of these genes with *phaC* only led to P(3HB) production in *E. coli*. The reason why only SCL PHA was produced could be that PhaC synthase from *Aeromonas caviae* preferred 3HB monomers or there was insufficient source of MCL 3HA monomers supplied from this pathway when glucose was added as a carbon source. Further genetically engineering *fabH* created a novel enzyme activity which was able to convert 3-ketoacyl-ACP into 3-ketoacyl-CoA (Nomura et al., 2004). The codon encoding the amino acid 87 of FabH protein sequence was modified to replace the Phe residue with various other residues by saturation point mutagenesis. The rationale of this work was that this modification would broaden enzyme substrate specificity toward longer-chain fatty acids since *M. tuberculosis* FabH with a Thr residue at position 87 displayed substrate specificity toward C_8 - C_{16}. Some of the point mutant *fabH* genes showed an increase of MCL-3HA fraction when co-expressed with either *Pseudomonas* sp. strain 61-3 *phaC1* gene or *A. caviae phaC* gene. The co-expression of engineered *fabH* gene with $phaC_{Ac}$ gene resulted in the incorporation of C_6 monomers into C_4 monomers, and obtained the highest fraction of C6 up to 5.8% with engineered FabH (F87C). Not only C_6 but also C_8 and C_{10} monomers were able to be detected in an *E. coli* strain harbouring engineered FabH and $PhaC_{Ps}$.

In another study, it was found that there is a link between the fatty acid β-oxidation pathway and the central metabolic pathway through an enzyme called thioesterase I (Test A) (Klinke et al., 1999). Therefore, PHA precursors could be obtained from non-related substrates such as sugars or gluconate through the fatty acid β-oxidation pathway. *E. coli* JMU193, a *fadB* mutant, was able to produce MCL PHAs consisting of C_6, C_8 and C_{10} up to 2.3% from gluconate by being introduced with a MCL PHA polymerase and the cytosolic thioesterase I.

1.8.1.2 PLA homopolymer, P(LA-*co*-3HB) copolymer and P(LA-*co*-3HB-3HV)/P(LA-*co*-3HB-*co*-3HHX) terpolymer

In the last decade and a half, PLA and a novel copolymer P(LA-*co*-3HB) has emerged as a promising bioplastic. This copolymer was first reported by a group of researchers from Japan in 2008 (Taguchi et al., 2008). This study triggered a series of subsequent studies to improve both polymer content as well as LA fraction (Jung et al., 2010b; Nduko et al., 2014; Shozui et al., 2011; Yamada et al., 2010; Yang et al., 2010; Yang et al., 2011). In the first report, Taguchi and colleagues successfully introduced LA monomer into P(3HB) to biosynthetically produce P(6 mol% LA-*co*- 94 mol% 3HB) copolymer by employing two enzymes propionate-CoA transferase (Pct) from *Clostridium propionicum* and engineered PHA polymerase (PhaC) from *Pseudomonas* sp. 61–3 in *E.coli* JM109. Not too long after, another group of researchers further investigated these two enzymes by using random mutagenesis of the pct_{cp} gene and site-directed mutagenesis as well as saturation mutagenesis of the $phaC1_{Ps6\text{-}19}$ gene (Yang et al., 2010). Three mutants (E130D, S325T, and Q481M) in the $phaC1_{Ps6\text{-}19}$ gene were generated to modify enzyme activity towards SCL 3HAs by using site-directed mutagenesis. Other modifications for the $phaC1_{Ps6\text{-}19}$ gene were performed using saturation mutagenesis to create a series of different combinations of mutated amino acids at the 310, 325, 481 and 477 sites. A wide range of LA

fractions from 20-49 mol% was obtained when these engineered enzymes were expressed in *E. coli*. An even wider range of LA fractions (9-64 mol%) could be achieved by fed-batch cultures. The effect of modification at these sites (E130, S325, S477 and Q481) was also investigated in other type II *Pseudomonas* PHA synthases 1 (PhaC1s) from *Pseudomonas chlororaphis*, *Pseudomonas* sp. 61-3, *Pseudomonas putida* KT2440, *Pseudomonas resinovorans* and *Pseudomonas aeruginosa* PAO1 (Yang et al., 2011). All PhaC1 that had mutated sites showed a change of substrate specificity towards SCL hydroxyl-CoAs consisting of lactyl-CoA and 3-hydroxybutyryl-CoA. The engineered *phaC1* gene from *Pseudomonas* sp. 61-3 was further improved to finely regulate the LA fraction in the copolymer by saturation mutation at position 392 (Yamada et al., 2010). By alignment, there is a correlation between site 392 in Type II PHA synthase from *Pseudomonas* sp. 61-3 and site 420 in the PHA synthase from *Ralstonia eutropha*. Previous studies showed that mutation at the position 420 in PHA synthase from *Ralstonia eutropha* enhanced the enzyme activity. Therefore, it could be interesting to examine the position 392 in engineered PHA synthase from *Pseudomonas* sp. 61-3. Out of the 19 mutants, there were 17 that exhibited the ability to incorporate LA with a range of various monomer composition (16 - 45 mol%).

In addition to enzyme activity, the culture conditions can have an effect on polymer production. It was found that anaerobic conditions could help increase LA monomer composition up 62 mol% (Yamada et al., 2010). Not only propionate-CoA transferase and PHA polymerase but also the host system was taken into account to produce the copolymer P(LA-*co*-3HB). Since *E. coli* is not a native polymer producer, it possesses other pathways that produce side products such as ethanol and acetate. In order to make it a robust host for polymer production, the *ackA*, *ppc* and *adhE* genes have been deleted, and the promoters of the *ldhA* and *acs* genes have been

replaced with the *trc* promoter based on in silico genome-scale metabolic flux analysis (Jung et al., 2010b). *E. coli* JLX10 which possesses all of the above engineered characteristics showed the most promising system for polymer production in terms of LA monomer composition and polymer content. The *pflA* mutant *E. coli* strain JW0885, which had a defective formate synthesis pathway from pyruvate, also increased LA fraction in the copolymer (26 mol%) (Shozui et al., 2010b). The cost-effective strategy which targeted the substrate used for P(LA-*co*-3HB) production was under consideration in a recent study (Nduko et al., 2014). Over-expresssion of a galactitol transporter (GatC), which partakes in the ATP-independent xylose uptake, was carried out in different mutant *E. coli* backgrounds. GatC over-expression resulted in an increase of both the LA fraction (67, 66 and 64 mol%) and polymer yield (8.3, 6.6 and 7.3 g/l) in mutants (*pflA, pta* and *dld* mutants, respectively).

Other terpolymer P(LA-*co*-3HB-*co*-3HV) and P(LA-*co*-3HB-*co*-3HHx) are novel polymers that have recently been discovered (Shozui et al., 2010a; Shozui et al., 2010b; Shozui et al., 2011). P(LA-co-3HB-3HV) terpolymer was first obtained in the *pflA* mutant *E. coli* strain JW0885 by expressing engineered PHA synthase from *Pseudomonas* sp 61-3 along with three other precursor supplying enzymes such as propionyl-CoA transferase, β-ketothiolase and acetoacetyl-CoA reductase (Shozui et al., 2010b). Feeding the recombinant strain with 2% glucose and 100 mg/l of sodium propionate resulted in the presence of 7.2 mol% LA monomer. The highest LA fraction was achieved up to 96 mol% in a P(LA-*co*-3HB-*co*-3HV) terpolymer (Shozui et al., 2011). In this study, propionyl-CoA transferase gene (*pct*) from *Megasphaera elsdenii*, the mutated PHA synthase gene from *Pseudomonas* sp. 61-3 (*phaC1STQK*), and the (R)-specific enoyl- CoA hydratase gene (*phaJ4*) from *Pseudomonas aeruginosa* were introduced into *E. coli* LS5218 to produce the flow toward PHA synthesis from glucose and valerate.

However, polymer content was only 0.4 wt% which is not economically efficient for commercialization. Another terpolymer P(LA-*co*-3HB-*co*-3HHx) was also discovered by the same group of researchers (Shozui et al., 2010a). Employing the same set of genes (*pct, phaC1STQK* and *phaJ4*) resulted in the incorporation of 3HHx through reverse reaction of β-oxidation pathway if the strain was fed with glucose and butyrate. Monomer composition consisted of LA (2.7 – 34 mol%), 3HB (38 – 81 mol%) and 3HHx (17 – 33 mol%) and varied depending on the concentration of sodium butyrate. However, polymer content is still quite low (<4%).

1.8.2 In yeast: *Saccharmyces cerevisae*

With the goal of improving PHA productivity as well as reducing the initial cost, other studies have considered yeasts as hosts to produce PHA. Also, studies of PHA formation in yeasts might provide valuable information about how peroxisomal metabolism needs to be modified to increase PHA production in other eukaryotes, such as plants. However, unlike prokaryotes which have been widely and intensively studied for PHA production, eukaryotes have still not yet attracted a lot of interests to be employed for PHA production due to the poor ability to synthesize and accumulate PHAs. In several studies, PHAs have been detected in low concentrations in yeasts compared to that in bacteria. *S. cerevisiae*, a model yeast widely used in the food industry, has been one of potential candidates for PHA production. P(3HB) synthesis genes in *C. necator* and *Methylobacterium extorquens* have been successfully introduced into *S. cerevisiae* (Breuer et al., 2002). Interestingly, expression of either PHA synthase gene or all three P(3HB) genes resulted in P(3HB) accumulation with P(3HB) content of 6.7% and 5.2%, respectively.

Not only P(3HB) but also MCL PHAs have been successfully produced in *S. cerevisiae*. *S. cerevisiae* was equipped with the *P. aeruginosa* PHAC1 synthase modified at the carboxyl end for peroxisome targeting (Poirier et al., 2001). This protein harbours 34 amino acids of the carboxy-terminal tripeptide ARM from *Brassica napus* isocitrate lyase. A wide range of even-chain PHA monomers from 6 to 14 carbons was generated by yeast grown on oleic acids. Meanwhile, various odd-chain monomers from 5 to 15 carbons were detected in yeast grown on heptadecenoic acid. The maximum PHA content was 0.45% when yeast was fed with oleic acid as a carbon source. Another peroxisome targeting signal employed to target PHA synthase to the peroxisome was the Ser-Arg-Met terminal tripeptide from the the isocitrate lyase enzyme (ICL) (Marchesini et al., 2003). Recombinant *S. cerevisiae* produced PHA containing even-chain monomers of between 6 and 12 carbons. Moreover, it was found out that both a functional peroxisome and β-oxidation pathway played an important role in yeast cells for PHA production. Scientists tried to produce PHA not only in yeast peroxisome but also yeast cytosol for improvement of PHA production. PHA production in cytosol is likely to benefit from a larger space which would not depend on the number and volume of peroxisomes, and from profuse carbon fluxes present during the growth. A detectable PHA amount has been achieved from cytosolic expression of the MCL PHA in wildtype and *pex5* mutant yeast which is deficient in the transport of perosixomal proteins with the PTS1 signal into organelle (Zhang et al., 2006). The maximum PHA content in yeast cytosol was 0.069 %DCW when *pex5* mutants were fed with both formate and dodecanoic acid.

The yeast β-oxidation cycle has been modified to channel carbon flux toward SCL-MCL PHA biosynthesis (Oliveira et al., 2004). The synthesis of SCL-MCL PHA copolymer containing 3 HAs of 4 and 6 carbons has been established in *S. cerevisiae* by the expression of two distinct

PHA synthases in yeast peroxisomes. PhaC synthases from *C. necator* ($PhaC_{Cn}$) and *Aeromonas caviae* ($PhaC_{Ac}$) were modified at the N termini by adding the first 16 amino acids from *S. cerevisiae* peroxisomal 3-ketothiolase protein, which help import foreign proteins into perosixome. *S. cerevisiae* strains deleted in 3-hydroxyacyl-CoA dehydrogenase were employed as hosts for SCL-MCL PHA production. To investigate the effect of different mutated 3 hydroxyacyl-CoA dehydrogenase, *fox2Δ* cells harbouring $PHAC_{Cn}$ or $PHAC_{Ac}$were reintroduced with wildtype and mutant Fox2. SCL-MCL PHA content significantly increased in *fox2Δ* cells harbouring $PHAC_{Cn}$/ $PHAC_{Ac}$ and mutant Fox2 in domain B (Fox2(bΔ)). Among them, *fox2Δ* cells harbouring $PHAC_{Cn}$ and Fox2(bΔ) accumulate PHA amount twice as much PHA as *fox2Δ* cells harbouring $PHAC_{Ac}$ and Fox2(bΔ). However, *fox2Δ* cells harbouring $PHAC_{Ac}$ and Fox2(bΔ) provided 6 times more 3-hydroxyhexanoic acid than *fox2Δ* cells harbouring $PHAC_{Cn}$ and Fox2(bΔ).

Because monomer composition determines favourable properties of PHAs, monomer modulation is still a question of great interest in addition to simply PHA content and PHA productivity. In one study, modification of the monomer composition of MCL PHA produced in *S. cerevisiae* has been carried out by the manipulation of the enzymes in the β-oxidation pathway (Marchesini et al., 2003). Variants of the peroxisomal multifunctional enzyme 2 (MFE-2) have been made by mutating A and B domains of the *S. cerevisiae* 3-hydroxyacyl-CoA dehydrogenase. MFE-2(aΔ) mutant having inactivated B domain showed a broad activity towards short (C4)-, medium (C10)-, and long (C16)- chain 3R-HA CoAs. Meanwhile, the MFE-2(bΔ) mutant having an inactivated B domain of the dehydrogenase preferred medium- and long-chain 3R-HA CoAs and did not accept short chain-3R-HA CoAs. Different substrates are likely to provide various types of PHA monomers including even-numbered, odd-numbered, or a

combination of even- and odd- numbered 3R-HAs. It was reported that the *pex5* mutant of *S. cerevisiae* grown on C13, C12, C11 and C10 fatty acids accumulated PHA monomers of C13, C12, C11 and C10 (approximately 45-77%), respectively (Zhang et al., 2006). Both even- and odd- chain PHA monomers were produced in the *pex5* mutant when undecanoic acid was used as the carbon source. When tetra decanoic acid and decanoic acid were fed in culture, yeast accumulated even-chain PHA monomer of C10, C8 and C6. Meanwhile, when tridecanoic acid and undecanoic acid were added into media, longer odd-chain monomers of C7 – C13 were obtained in yeast cytosol.

1.8.3 In Plants: *Arabidopsis thaliana* and *Alfalfa* (*Medicago sativa*)

In addition to bacteria and yeasts, plants were also considered for being a host of biopolymer production due to the fact that plants can produce carbon source and obtain most of their energy from sunlight via photosynthesis (Figure 1.6). Acetyl-CoA, which is an essential precursor to the PHA production pathway, is widely distributed in plant cells including the cytoplasm, plastid, mitochondria and peroxisome (Yunus et al., 2008). It is interesting that there has been a wide diversity of plants that were used to study polymer production such as *Arabidopsis thaliana*, *Tobacco (Nicotiana tabacum)*, *Rapeseed (Brassica napus)*, *Cotton (Gossypium hirsutum)*, *Alfalfa (Medicago sativa)*, *Flax (Linum usitatissimum)* and *Oil palms (Elaeis guineensis and E. oleifera).* Discussed in more detail below are two representative plants that were selected to demonstrate the ability for polymer production: *Arabidopsis thaliana* and *Alfafa (Medicago sativa).* The reason is that *Arabidopsis* is a model plant that has intensively been studied, and this plant also was the most successful plant system to produce biopolymer in terms of polymer content as well as the variety of polymer types. The second selected plant,

Alfafa, was known to have a symbiotic relationship with *S. meliloti* which is the main host used in this study.

1.7.3.1 *Arabidopsis thaliana*

The first plant that was used for PHA production is *Arabidopsis thaliana* because this plant is considered as a model plant to study plant structure and function. The cytoplasm initially appears to be the most appropriate subcellular compartment because genes do not need any modification to be expressed in this compartment, and one of essential enzymes involved in P(3HB) production, 3-ketothiolase, is already present in the cytosol as part of the mevalonate pathway. However, expressing two genes *phaB* and *phaC* from *C. necator* in *A. thaliana* by cross-pollination led to only 0.1% of P(3HB) produced in the leaf tissue (Poirier, 2002). Interestingly, P(3HB) was found not only in cytoplasm but also in other compartments such as the vacuole and nucleus, with the exception of the plastids and mitochondria whose double membrane might prevent P(3HB) from penetrating into these sites. The next compartment to be targeted for PHA production is the plastid, which provides a high flux of carbon and a closed space to separate P(3HB) and protect other cell components. In order to facilitate P(3HB) production in this compartment, three genes including *phaA*, *phaB* and *phaC* need to be modified at the N-teriminus with the transit peptide from the small subunit of Rubisco under the *CaMV35S* promoter so that these gene products are imported and able to function well within the plastids. P(3HB) production in these transgenic plants was shown to be much improved up to 14% DCW (Nawrath et al., 1994). However the transgenic plants expressing these plastid-targeted genes which were obtained by cross-pollination seem unstable. To counteract this, other groups developed a transformation vector that contains all the genes which then can be integrated into one single site of the plant genome. This led to P(3HB) accumulation up to 13% and 40% DCW

reported by Valentin et al. (1999) and Bohmert et al. (2000), respectively. The plant growth, however, was severely stunted with high P(3HB) accumulation. Analysis of various metabolites in plants during P(3HB) accumulation indicated that the TCA cycle was affected, but not the fatty acid pool.

Arabidopsis was also engineered to produce P(3HB-*co*-3HV) (Slater et al., 1999; Valentin et al., 1999). In order to produce this type of polymer, the plant was transformed with four genes: one gene from *E. coli* (*ilvA*) and three genes from *C. necator* (*bktB*, *phbB*, *phbC*). The 3-HV precursor was derived from threonine via threonine deaminase and the pyruvate dehydrogenase complex. A tool which helps to predict the production of this copolymer in plants was constructed and published in 1999 (Daae et al., 1999). The light condition greatly influenced the monomer composition as well as the copolymer production rate. Among the enzymes, β-ketothiolase played a crucial role in controlling the copolymer rate while all the enzymes determined the copolymer ratio. β-ketothiolase increased resulting in the increased flux and reduced 3HV/3HB ratio in the polymer, while acetoacetyl-CoA reductase and P(3HB) synthase increased resulting in the increase of both the flux and the ratio.

1.7.3.1 *Alfalfa (Medicago sativa)*

Alfafa was considered as a host to produce P(3HB) because it was known to generate a great deal of biomass for energy uses (Saruul et al., 2002; Yunus et al., 2008). It is a source of forage and animal feed. This plant also has a symbiotic relationship with *S. meliloti*, which is the main host used for polymer production in this study. The plant was targeted for the production of either P(3HB) or P(3HB-*co*-3HV) in the leaves by being introduced to three plastid-targeting genes involved in P(3HB) synthesis (*phbA, phbB, phbC*) or P(3HB-*co*-3HV) synthesis (*bktB,*

phbB, phbC). However, the P(3HB) content was extremely low and fluctuating from 0.025 to 1.8 g/kg DCW. Moreover, P(3HB-*co*-3HV) was unable to be obtained in this plant due to insufficient flux of substrates to synthesize β-ketoval-CoA. Instead, only P(3HB) was obtained in the transgenic plant which carries the *bktB*, *phbB*, *phbC* genes with a lower amount compared to the plant carrying *phbA*, *phbB*, *phbC*. Therefore, the P(3HB) amount was dependent on which specific β-ketothiolase gene (*phbA* or *bktB*) was used. The only bright side was that there was no issue related to stunted growth. Additionally, similar P(3HB) amount was observed between F1 hybrid progeny and parental line suggesting that polymer production in alfafa was a stable and dominantly inherited trait.

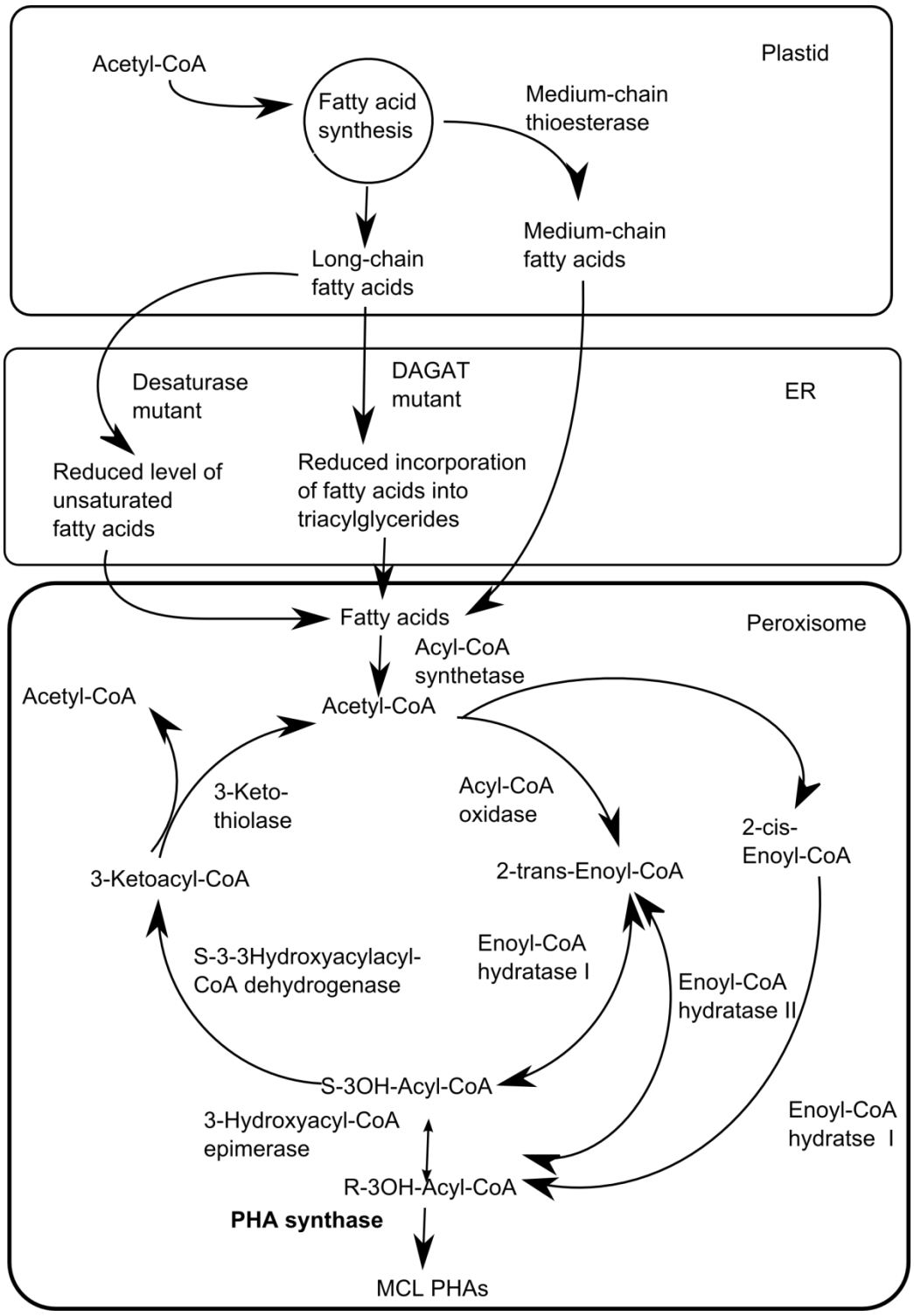


Figure 1.6 Modification of plant metabolic pathways for the synthesis of medium-chain-length polyhydroxyalkanoate (MCL-PHA) in the peroxisomes of plants (Adapted from Poirier, 2002)

1.9 Advanced applications of PHA

PHAs are not only biodegradable materials which may replace petroleum-derived plastics but they also be employed for advanced applications in medical and biological fields. Biocompatibility is one of the most important properties for the materials to be considered for health-related applications such as implantation and drug carriers. Since side effects might lead to lethal consequences when using traditional materials, such as silicone and synthetics to construct artificial human tissue, it is desirable to discover materials having a good quality as well as available biocompatibility (Jung et al., 2010a). Therefore, PHA has been found to be a preferable choice in tissue engineering including scaffold development (Park et al., 2005a). Blends of hydroxyapatite (HAP) and P(3HB)/P(3HB-*co*-3HHx) were evaluated in the application of making scaffold for bone reconstruction. It was interesting that different effects on mechanical properties of scaffolds and osteoblast response were obtained when HAP was incorporated into different kinds of PHA; incorporation of HAP into P(3HB) improved osteoblast response, while its incorporation into P(3HB-*co*-3HHx) had an adverse effect (Wang et al., 2005). Likewise, different effects on the cell were also observed in scaffold development depending on the specific blend of PHAs. Chondrocytes grew and proliferated best on PHAs that were made from the blend of P(3HB-*co*-HHx) and P(3HB) at the ration of 6:4 (Zhao et al., 2003).

In addition to scaffolds in tissue engineering, PHAs have been used in various medical fields, such as vascular grafts, stents, patches, sutures and drug carriers. In particular, P(3HB) micropheres were employed for sustained drug release. However, it takes longer for P(3HB) to degrade in the body compared to other commercial polymers, such as polyglycolide (PGA), due to its high crystallinity. Therefore, an alternative material with more flexibility and less crystallinity, P(3HB-*co*-3HV), has been taken into consideration for applications related to drug

carriers in microspheres and implants. P(3HB-*co*-3HV)-coated microspheres and microcapsules were designed to contain tetracycline and examined for encapsulation efficiency, loading, release characteristics, and morphological properties (Sendil et al., 1999). To improve the encapsulation efficiency and prolong the release period, it was neutralized with NaOH. Biodegradability was not an issue in the release behavior because the release process was completed much before than the beginning of polymer degradation. In addition, implantation of antibiotic-loaded rods of P(3HB-*co*-3HV) for the treatment of chronic osteomyelitis was examined by introducing the rods into a rabbit tibia artificially infected by *Staphylococcus aureus*, and complete recovery was observed in 30 days (Yagmurlu et al., 1999).

PHAs were also applied in immunoassays based on the substrate specificity of PHA depolymerase (Lee et al., 2005). In this study, two model detectable proteins were used including enhanced green fluorescent protein (EGFP) from *Aequorea victoria* and red fluorescent protein (RFP) from *Discosoma*. At the C-terminus, EGFP or RFP was fused to the substrate-binding domain (SBD), a part of PHA depolymerase used to recognize PHA and bind to it. At the N-terminus, model proteins were tagged with a hexahistidine for downstream purification using Ni columns. The mean fluorescence from protein-binding PHA microbeads was 3-fold higher than the negative control. This indicated that both specific protein-binding interaction and post-binding biologically active fusion proteins were achieved. For an example of a practical application, this approach has been employed to design an immunoassay system for severe acute respiratory syndrome (SARS) virus detection. A fusion protein composed of the SARS coronavirus (SARS-CoV) envelope protein (SCVe) and SBD was constructed and produced in recombinant *E. coli*. This fusion protein was used to raise the anti-SCVe rabbit polyclonal antibody. Then the fusion protein was incubated with PHB-coated microbeads which

subsequently came in contact with anti-SCVe antibodies. After the unbound protein was washed away, the binding event was determined using flow cytometry. As expected, the results demonstrated that the fluorescence signal was detected only when the binding event occurred. The follow-up study examined the application of using PHA-coated slides to detect the hepatitis B virus (HBV) preS2 surface protein and SCVe (Park et al., 2006). Likewise, the single-chain antibody (ScFv) against HBV protein and SCVe were fused to SBD before being fixed on PHA-coated slides via microspotting. Fluorescence was only detected upon specific antigen-antibody interaction, indicating that this technique can be considered as a useful method in immunoassay with a high degree of affinity and selectivity.

Another study demonstrated PHA synthases can be used as molecular tools to recycle biocatalysts by covalently immobilizing the enzyme at the PHA granule surface. The β-galactosidase (LacZ) was fused to the N-terminus of PHA synthase (PhaC1) from *P. aeruginosa* via a linker region. By introducing the open reading frame of *lacZ-phaC1*, *P. aeruginosa* PHA-negative mutant strain could restore the ability of PHA biosynthesis, showing a PHA synthase activity from the fusion protein. The surface of PHA granules retained more LacZ-PhaC1 fusion proteins than PhaC1 alone. The treatment of these LacZ-PHA granules with urea suggested a covalent binding of the LacZ-PhaC1 fusion protein to the PHA granule. Moreover, the immobilized LacZ remained stable for several months under various storage conditions, with its own activity. Therefore, a powerful platform for efficient *in vivo* enzyme immobilization was developed by using PHA and engineered PHA synthase (Peters and Rehm, 2006).

Application of PHAs has also been used in water and wastewater treatment, specifically the denitrification step (Hiraishi and Khan, 2003). Traditionally, in order to remove nitrogen, either organic matter or simple organic compounds (e.g., acetate and methanol) were used as

electron donors in the system. However, both of them had their own drawbacks that required some alternative solution to replace this conventional system. Researchers developed the solid-phase denitrification process using PHAs and related biodegradable polymers as a constant sources of reducing power since PHAs easily degraded in the system. Additionally PHAs are insoluble in water, hence they can also serve as solid matrices for biofilm development which help control the process rate.

A new trend of PHA applications is the production of PHA-derived biofuels (Zhang et al., 2009). SCL and MCL PHAs were cleaved and converted into methyl esters (ME) via the methanolysis reaction. The entire process is quite straight-forward and feasible in a single fermenter as shown in Figure 1.7. Combustion heat generated from 3HBME and 3HAME were 20 and 30 kJ/g, respectively, while ethanol generated a combustion heat of 27 kJ/g. Interestingly, the combustion heat of ethanol could be increased up to 30 and 35 kJ/g by adding 10% 3HBME or 3HAME. The cost to produce PHA-derived biofuel was roughly estimated around US$1200.

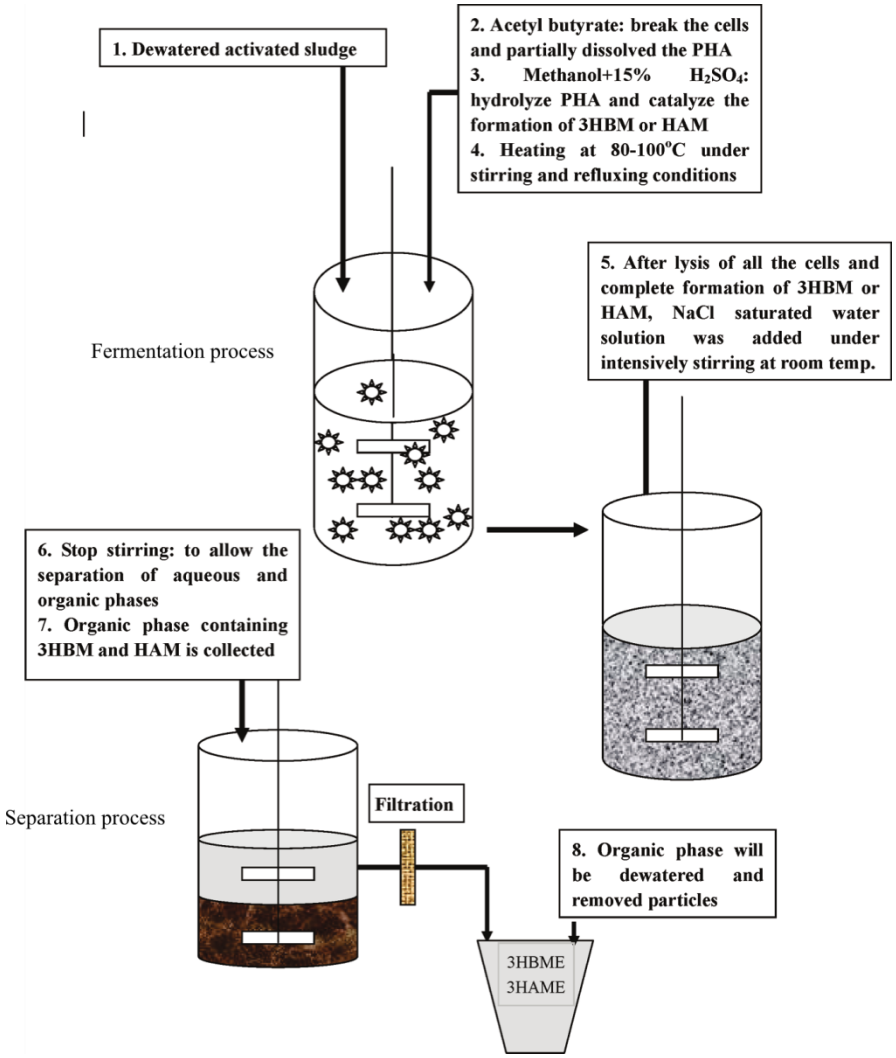


Figure 1.7 Schematic illustration of production PHA-based biofuels (Adapted from Zhang et al., 2009)

1.10 Objectives of this study

This study aimed to engineer bacterial strains so that they are able to produce a broad range of bioplastic polymers such as PLA, P(3HB-*co*-LA), and P(3HB-3HHx-3HO-LA) with different properties for various applications. The primary rationale for choosing *S. meliloti* and *P. putida* strains is that these strains are native SCL and MCL PHA producers, respectively, and they are also genetically amenable. In both cases, complete genome sequences are available, and the PHA synthesis genes are known. Also these strains are able to use a broad range of substrates. For example, *P. putida* is able to use both fatty acids and sugars to produce MCL PHAs. The strains also can grow rapidly, reach high titre, and are able to readily accumulate polymer granules.

For novel polymer production, recent studies have mostly focused on engineering *E. coli* strains that are common model systems in the metabolic engineering field. *E. coli* strains have been engineered to produce a broad range of bioproducts such as biopolymers, biofuels, amino acids, and succinic acid because their molecular tools are intensively studied and widely available. However, *E. coli* strains are not native polymer producers; hence, they lack the system for the production of usable polymer precursors. Also, it was shown that *E. coli* strains suffer from the stress of polymer production, and they produce some side products that might decrease the overall yield of target products. For these reasons, we focused our study on engineering these two representative native polymer producers for an efficient novel polymer production system. Our approach is to introduce two codon-optimized engineered genes encoding propionate CoA transferase (*pct*) and PhaC synthase (*phaC*) into the aforementioned hosts. Based on previous studies, the novel polymer P(3HB-*co*-LA) was able to be produced in *E. coli*, a non-native producer by introducing these two engineered enzymes (Jung et al., 2010b). The *pct* gene was

engineered to accept lactate as a substrate and convert it into (D)-lactyl-CoA in the cell, while the *phaC* gene was engineered to broaden the substrate specificity toward SCL, PHA and lactate.

For the *S. meliloti* host, two strategies were outlined; one involved integrating the genes into the chromosome and expressing them under the native *phbC* promoter, or so-called chromosome-engineering; the other is putting the genes in an expression vector and expressing them under an inducible *tac* promoter. The advantages of the first strategy are that the genes remain more stable and fixed on the chromosome with less risk of being lost during production; also, putting them under native promoter should result in expression of the genes in a manner similar to the expression of the native *phbC* gene. The advantages of the second strategy are that the genes can be expressed under a tunable promoter; moreover, this system is more flexible and can be easily introduced into different hosts for a broader range of application in term of the types as well as the properties of the produced polymer. For *P. putida* host, the initial investigation was carried out when the genes were expressed on the plasmid-based form. As mentioned earlier, the plasmid-based system is very flexible; therefore, transferring this system into *P. putida* can be done easily in a short period of time. Another aspect to explore is promoter engineering which involves replacing the inducible promoter with a broad range of native promoters of different strength.

Chapter 2 Material and methods

2.1 Bacteria, primers and growth conditions

2.1.1 Bacteria and primers used

All strains and plasmids used in this study are listed in Table 2.1. The strains were stored at -80ºC in Tryptone Yeast extract broth with 7% DMSO.

2.1.2 Media and growth condition

E. coli and *P. putida* were routinely cultured in Luria-Bertani (LB) media while *S. meliloti* was cultured in Tryptone Yeast (TY) media, supplemented with appropriate antibiotics as necessary for plasmid maintenance (Charles and Finan, 1990; Charles and Finan, 1991). Suicide plasmid pK19*pctPhaC* was maintained in *E. coli* using Km (25 µg/mL). Expression plasmid pTH1227 and its derivatives were maintained in all strains using tetracycline (10 µg/mL). The recipes of all media can be found in the Appendix.

For polymer production in *S. meliloti*, strains were initially inoculated in TY broth, and then 1% overnight culture was transferred to Yeast Extract Mannitol (YEM) media in flasks on a shaker at 180 rpm, 30C for 3 days. IPTG at 0.4 mM was added into the culture for induction, unless otherwise stated. Rapid screening of polymer production was performed by adding Nile Red (0.5 µg/mL) to YM agar plate, and observing its mucoid phenotype or visualizing by fluorescence (Heil et al., 2011).

For polymer production in *P. putida*, strains were initially inoculated in LB broth, and then 1% overnight culture was transferred to either LB or defined media (0.5E2) in flasks on a

shaker at 180 rpm, 30°C for 3 days. Nile Red (0.5 µg/mL) was added to LB or 0.5E2 agar plate for rapid screening of polymer production.

Table 2.1 List of strains that were used in this study

Strains or plasmids	Genotype	Reference
E. coli		
DH5α	*supE44 lacU169(w80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(MacLellan et al., 2006)
DH5α(pRK600)	Helper strain harbouring plasmid pRK600	(Finan et al., 1986)
S. meliloti		
Rm1021	Spontaneous Sm isolate of *R. meliloti* SU47	(MacLellan et al., 2006)
Rm11144	Rm1021 *phaC1*::Tn5-233	(Charles et al., 1997)
Rm7055	Rm1021 *exoF*:: Tn5	(Leigh et al., 1985)
SmUW499	Rm1021 *ΔphaC exoF*:: Tn5	Ricardo Nordeste
SmUW501	Rm1021 *ΔphaAB ΔphaC exoF*:: Tn5	Ricardo Nordeste
SmUW254	Rm1021 *phaC1*::*pct PhaC*	This study
SmUW255	SmUW499 harbouring pTH1227	This study
SmUW256	SmUW499 harbouring pTAM	This study
SmUW257	Rm7055 harbouring pTH1227	This study
SmUW498	Rm1021 *ΔphaAB exoF*:: Tn5	Ricardo Nordeste
SmUW502	Rm1021 *ΔphaAB ΔphaZ exoF*:: Tn5	Ricardo Nordeste
SmUW503	Rm1021 *ΔphaC ΔphaZ exoF*:: Tn5	Ricardo Nordeste
SmUW500	Rm1021 *ΔphaAB ΔphaC exoF*:: Tn5	Ricardo Nordeste
SmUW550	Rm1021 *ΔphaAB exoF*:: Tn5 harbouring pTH1227	This study
SmUW551	Rm1021 *ΔphaAB exoF*:: Tn5 harbouring pTAM	This study
SmUW552	Rm1021 *ΔphaAB ΔphaZ exoF*:: Tn5 harbouring pTH1227	This study

SmUW553	Rm1021 *ΔphaAB ΔphaZ exoF*:: Tn5 harbouring pTAM	This study
SmUW554	Rm1021 *ΔphaC ΔphaZ exoF*:: Tn5 harbouring pTH1227	This study
SmUW555	Rm1021 *ΔphaC ΔphaZ exoF*:: Tn5 harbouring pTAM	This study
SmUW556	Rm1021 *ΔphaZ exoF*:: Tn5 harbouring pTH1227	This study
SmUW557	Rm1021 *ΔphaZ exoF*:: Tn5 harbouring pTAM	This study
SmUW558	Rm1021 *ΔphaAB ΔphaC exoF*:: Tn5 harbouring pTH1227	This study
SmUW559	Rm1021 *ΔphaAB ΔphaC exoF*:: Tn5 harbouring pTAM	This study
SmUW560	SmUW499 harbouring pTAM2	This study
SmUW561	SmUW499 harbouring pTAM3	This study
SmUW562	SmUW499 harbouring pTAM4	This study
SmUW563	SmUW499 harbouring pTAM5	This study
SmUW564	SmUW499 harbouring pTAM6	This study
SmUW565	SmUW499 harbouring pTAM7	This study
SmUW567	SmUW499 harbouring pTAM2noinsert	This study
SmUW568	SmUW499 harbouring pTAM6noinsert	This study
SmUW569	SmUW499 harbouring pTAM7noinsert	This study
P. putida		
PPUW1	*P. putida* KT2440 (Rif^r)	(Le Meur et al., 2012)
PPUW2	Δ*phaC1-phaZ-phaC2*/ ΩKm in PPUW1(Km^r)	Jiujun Cheng
PPUW18	PPUW2 harbouring pTH1227	This study
PPUW19	PPUW2 harbouring pTAM	This study
PPUW20	PPUW1 harbouring pTH1227	This study
PPUW21	PPUW1 harbouring pTAM	This study

Table 2.2 List of plasmids and primers that were used in this study

Plasmids	Description	Reference
pRK600	pRK2013 npt::Tn9 Cm^r Nm-Km^s	(Finan et al., 1986)
pTH1227	pFUS1 carrying *lacI*^{q}-P_{tac} DNA from pMal-c2x (AB32193/AB32194), Tc^r	(Cheng et al., 2007)
pTAM	pTH1227 *pct532 phaC1400* (codon-optimized)	This study
pK19*mobsacB*	suicide plasmid mobilizable (*oriT*, *oriV*) *sacB lacZα*, Km^r	(Schäfer et al., 1994)
pK19*pctphaC*	pK19*mobsacB* derivatives carrying *pctphaC* fragment	This study
pTAM2	pTAM derivative, P_0 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM3	pTAM derivative, P_1 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM4	pTAM derivative, P_2 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM5	pTAM derivative, P_3 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM6	pTAM derivative, P_4 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM7	pTAM derivative, P_5 promoter- a substitute for *lacI*^{q}-P_{tac}	This study
pTAM2-no*pctphaC*	pTAM2 derivative, removal of *pctPhaC* fragment	This study
pTAM6-no*pctphaC*	pTAM6 derivative, removal of *pctPhaC* fragment	This study
pTAM7-no*pctphaC*	pTAM7 derivative, removal of *pctPhaC* fragment	This study
pUC57*pctphaC*	pUC57 harbouring two genes, *pct* and *phaC*	This study
pSRKGm	pBBR1MCS-5-derived broad-host-range expression vector containing *lac* promoter and *lacI*^{q}, *lacZα*$^+$, and Gm^r	(Khan et al., 2008)
pSRK*ldhA*	pSRKGm carrying *ldhA* gene from *E. coli*	This study
pSRK*ldhAacs*	pSRK*ldhA* carrying *acs* gene from *E. coli*	This study

pSRK*Sfacs*	pSRKGm carrying *acs* gene from *S. fredii*	This study
pSRK*glcDacs*	pSRK*Sfacs* carrying *glcD* gene from *S. fredii*	This study
pJet*Ecacs*	pJet carring *acs* gene from *E. coli*	This study
pTH1227a	DH5α harbouring	This study
pTH1227b	DH5α harbouring pTH1227b	This study

Table 2.3 List of native promoters, their sequences and original genes which were expressed under these promoters in *S. meliloti*

Promoter name	Promoter sequence	Genes involved
P_0	5’-aagcttTCTAGAGTTGACAGGACGAATGCAGGCGGTCAT ATACCCGGCGCAGActcgag-3’	*smc1378*
P_1	5’-ctagaAGATCTGTTGACACGATTGGGTCGACACGGTATG TGCCTGCCTACTTc-3’	*rpmJ*
P_2	5’-ctagaAGATCTCTTGTTATCCCGCAGACCTTCATGTATAG Gc-3’	*rpmE*
P_3	5’-ctagaAGATCTCTTGTGGTTTTCGCAATCGGTCTTTATGTA GGGTCCAAACAc-3’	*secE*
P_4	5’-ctagaAGATCTCTTGACGGGATGAAAAATTCTGGGAATCAC CATTTCAAGCAc-3’	*rpoD*
P_5	5’-ctagaAGATCTCTTGAGATTCCTCATTTCCTGATCAATTTC GGGTCAACCGGc-3’	*ropB1*

2.2 DNA manipulation

2.2.1 Plasmid isolation

Plasmid minipreps from *E. coli* were carried out using an alkaline lysis method (Birnboim and Doly, 1979). All the components in all solutions can be found in the Appendix. The cell pellet of 3-5 ml overnight culture was harvested by centrifuging at 13,000 rpm, 30 sec. Then the cell pellet was re-suspended in 250 µl of resuspension solution I, followed by adding 250 µl lysis solution II and inverting the tubes several times to lyse the cell membrane and release DNA and protein into the solution. The next step is to precipitate out proteins and chromosome DNA by adding 350 µl of neutralization solution III, inverting the tubes and centrifuging at 13,000 rpm, 5 min. Now the precipitates settle at the bottom of the tubes, and the lysate can be removed by pipetting and transferred to the commercial BioBasic silica spin columns. Subsequently, plasmid DNA can be collected on the column by centrifuging these columns and removing the liquid passing through. The following step is the washing step by adding 500 µl of washing buffer into the column and centrifuging the column again. Trace amount of ethanol in washing buffer can be completely removed to avoid interfering the subsequent enzyme reaction by centrifuging the column once again. DNA plasmid can be eluted in 50 µl of elution buffer.

2.2.2 Genomic isolation

This method was adapted from a previous study (Meade et al., 1982). A 5-ml volume of culture was harvested by centrifuging at 13,000 rpm, 1 min. Then the liquid was discarded. The cell pellet was resuspended in 500 µl TES (10 mM Tris, 25 mM EDTA, pH 8.0) and then lysed by the addition of lysis solution which was composed of 20 µl of 25% SDS, 50 µl of 5mg/ml of predigested Pronase E and 50 µl of 5 M NaCl. The lysis occurred at 68°C, 30 min, followed by

the addition of 260 µl of 7.5 M ammonium acetate. Proteins were precipitated out when the mixture was left on ice for 20 min. Proteins were then removed by centrifuging at 13,000 rpm for 15 mins. DNA was extracted using chloroform in a ratio 1:1 after the tubes were inverted several times. Liquid on the top phase was transferred to a new tube containing 780 µl of isopropanol. The tubes were incubated on ice for 30 min to precipitate DNA. DNA was pelleted at the bottom of the tube by centrifuging 13,000 rpm, 15 min and removing the supernatant. The DNA pellet was washed with 500 µl of 70% ethanol before centrifuging at 13,000 rpm for 1 min. After the supernatant was discarded, DNA was dissolved in 50 µl of $T_{10}E_1$ buffer.

2.2.3 Enzyme digestion

DNA was digested using the appropriate enzymes (Thermo Scientific). The reaction mix are composed of samples, 0.5 µl of the appropriate enzymes and 1X Fast Digest reaction buffer in a total volume of 10 µl. This mixture was then incubated at 37°C, 30 mins.

2.2.4 Dephosphorylation of vector DNA

To prevent the backbone vector from re-circularizing, dephosphorylation was carried out using Fermentas shrimp alkaline phosphatase (SAP). The reaction mixture consisted of the digested vector, 1X reaction buffer and SAP. The mixture was then incubated at 37ºC, 10 min followed by the inactivation of SAP at 65°C, 5 min.

2.2.5 Ligation

The molar ratio of the backbone to the insert was normally 1:3. Then 0.5 µl of ligase and 1X ligase buffer were added into the mixture, followed by overnight incubation at 16°C. Negative control was always included, which contained sterile DW instead of the insert.

2.2.6 Agarose gel electrophoresis

0.8% agarose gel was made in 1X TAE buffer and casted on the tray. Samples were loaded into the wells and ran at 110V for roughly 50 min. Fermentas Generuler 1 kb DNA ladder was also run along with samples. DNA was visualized using a UV box composed of a dual intensity UV transilluminator and a camera connected to a computer.

2.2.7 Gel purification

Samples were loaded and run on 0.8% agarose gel for 1 hr. By being visualized under UV, the desired band was then cut out and weighted. Then the gel was dissolved in 3 volumes of binding buffer II by being incubated at 65ºC for 5 min or until the gel was dissolved completely. The tubes were inverted several times to mix the solution, and the solution was subsequently loaded onto commercial Biobasic silica spin columns and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded, while the DNA samples were remained on the filters of the columns. And these columns were washed with 500 µl of the washing buffer and dried by centrifuge. 50 µl of elution was the added into the column to elute DNA into a new microcentrifuge tube.

2.2.8 High-fidelity PCR

To amplify and clone desired genes, the high-fidelity polymerase KOD Hotstart, purchased from Novagen, was used to minimize amplification errors. The total reaction volume was normally 50 µl including 1 µl of template (10-100 ng/µl) , 1.5 µl of 100 µM each primer (forward and reverse), 5 µl of 2 mM dNTP, 25 µl of 2X Xtreme buffer, 1 µl of KOD and water topped up to 50 µl.

PCR was set following the normal procedure: Initialization step: 94°C for 2 min (1 cycle); Denature step: 94°C for 20-30 sec, Annealing step: temperature was adjusted according to used primers (normally 5°C lower than melting temperature) for 30 sec, Elongation step: 68°C (time depending on the length of amplified DNA with the rate of 1 kb/min) (these three step were repeated for 25-30 cycles); Final elongation: 68°C for 5 min (1cycle); Final hold: 4°C.

2.2.9 Colony PCR

To confirm the presence of target DNA, colony PCR was performed using either PCR master mix purchased from Thermo Scientific or home-made Taq polymerase.

For Taq polymerase, the reaction mixture included 1 µl of 100 µM each primer (forward and reverse), dNTP, 0.5 µl of Taq polymerase, 2 µl of 10X Taq buffer, 2 µl of 25 mM MgCl2 (optional), DMSO (optional if DNA was GC-rich) and water topped up to 20 µl. If there were multiple reactions of the same type, the Master mix was prepared before aliquoting into PCR tubes.

For PCR master mix, all components were supplied in the mix purchased from Thermo Scientific. Primers were added into the mix with the same amount as previously mentioned. Template is the colony picked up by yellow tips and mixed in the reaction mixture.

Colony PCR was carried out using the normal PCR protocol or touch-down protocol. The normal PCR is similar to the high-fidelity PCR protocol except for initialization step (95°C, 3-5min) and elongation (72°C). Touch-down protocol was set as following: Initialization step 95°C, 3-5min (1cycle), Denature step: 94°C for 20-30 sec, Annealing step: temperature was set at the initial temperature 70°C and decreased 0.5°C for every subsequent cycle, 30 sec for each

cycle, Elongation step: 72°C (time depending on the length of amplified DNA with the rate of 1 kb/min) (these three step were repeated for 25-30 cycles); Final elongation: 68°C for 5 min (1cycle); Final hold: 4°C.

2.3 DNA transferring

2.3.1 Heat-shock transformation

E. coli strain DH5α was used for cloning throughout the project. Heat-shock transformation was first carried out by adding ligation mix into competent cells. Then the competent cell mixture was left sitting on ice for 30 min before moving to the heat block at 42ºC for 90 sec. After doing heat-shock transformation at 42ºC to transfer DNA into the cells, the mixture stayed in ice for 5 min. Subsequently, 1 ml LB was added into the mixture which would be cultivated at 37ºC for 1 h to recover DNA-containing cells. Transformants were selected on LB plates containing the appropriate antibiotics.

2.3.2 Conjugation

1 ml of recipient, helper and donor strains were spun down using centrifuge at 13000 rpm, 30 sec. 500 µl of saline (5% NaCl) was then used to wash these cell pellets twice. Recipient, helper and donor strains were then re-suspended in saline and mixed together in the ration of 2:1:2. Then the mixture was spotted on TY media and incubated at 30ºC, overnight. These mating spots were subsequently streaked on selection plates using the appropriate antibiotics.

2.3.3 Transduction

The lysate was diluted in 1/20 or 1/50 before 1 ml of diluted lysate was mixed with 1 ml of the recipient culture. The mixture was kept at room temperature for 20 mins. The cell were

pelleted and washed twice with saline by centrifuging at 13,000 rpm, 30 secs, followed by being re-suspended in saline. The cell mixture was then plated on LB with the appropriate antibiotics and incubated at 30ºC.

2.4 RNA work

2.4.1 RNA isolation

RNases are present everywhere and very active, hence extra care must be taken to avoid sample degradation by using gloves and RNase-free materials. All solutions are prepared in RNase-free water or DEPC-treated water. Cells were sub-cultured in TY and grown to OD = 0.5 – 0.8. Stop solution which is 5% phenol in ethanol was added into the culture and mixed well to stop the cell growth. Next, culture was harvested by centrifuging at 5,000 rpm, 4°C for 10 min. Cells can be stored at -70°C after flash freezing in liquid nitrogen.

RNA extraction was performed following the hot phenol extraction protocol with some modification (Lin-Chao and Bremer, 1986). All the following steps should be carried out at 4°C, unless otherwise stated. The cell pellet from a 50 ml culture was thawed on ice and re-suspended in 960 µl of RNase-free water by vortexing. Next, 480 µl of hot phenol solution was added to an equal volume of cell re-suspension, vortexed vigorously and incubated in a water bath at 95°C for 1 min. The sample was spun down at 13000 rpm, 4°C for 10 min. Supernatant was added to 600 µl phenol/chloroform, and vortexed vigorously. Then, it was spun again for 5 mins, and aqueous phase was extracted twice with chloroform. To precipitate nucleic acid, the aqueous phase was added to 1/10 volume of sodium acetate and 2 volume of isopropyl alcohol, inverted to mix and incubated on ice for 30 min. Next, the sample was spun for 10min, washed with -20ºC 70% ethanol and air-dried until the pellet turned translucent. Precipitate was then

resuspended in 85 µl water and digested with 5 µl DNase I in 10 µl DNase I buffer at 37°C for 30 min to remove DNA from the sample. The DNA-free sample was confirmed by running on an agarose gel. If DNA is still present in the sample, water was added into the sample up to the volume of 400 µl, and nucleic acid precipitation was repeated as described above, followed by another round of DNA digestion. The RNA sample was further purified using Qiagen RNeasy mini kit, and finally resuspended in 100 µl water. The quantification of RNA was determined using the Nanodrop, and the ratio of A_{260}/A_{280} should be around 2.0. The quality of RNA was checked by imaging the agarose-formaldehyde gel to observe rRNA bands.

2.4.2 Dot blot

Samples with equal amount of RNA were applied onto a positively charged membrane using the Bio-Dot Microfiltration Apparatus (Bio-Rad). The membrane was placed on the gasket and wetted with 2x SSC. Saran wrap was used to cover unused wells. RNA samples were blotted to each well, followed by washing with 200 µl TE. Vacuum was used to help liquid pass through the membrane. Membrane fixation was performed using the UV-crosslinker instrument, using C-L program. Prehybridization was performed in DIG Easy Hyb buffer (Roche) at 40ºC for 30-45 min, followed by hybridization overnight at the same temperature. Washing and detection steps were carried out as outlined in the manufacturer's protocol for the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

2.5 Enzyme-related works

2.5.1 β-glucuronidase activity assay

This assay was carried out following the procedure as previously described (Russell and Klaenhammer, 2001). Cells were fully grown in TY media, and then the culture was added into the assay buffer at the ratio of 1:4, incubating at room temperature until the mixture turned into a

yellow color, at which point sodium carbonate was added to terminate the reaction. Reaction time and the absorbance of the mixture at 420 nm were recorded. Cell density of the culture was measured at 600nm for normalization.

2.6 Cell disruption methods

2.6.1 Bead-beater

5 ml of culture was harvested by centrifuging at 13,000 rpm, 30 sec. Then the cell were washed with 1 ml of lysis buffer and harvested again. The supernatant was discarded, while the cells were resuspended in 500 µl sonication buffer. Roughly 350 µl of 0.1 mm silica beads was added to the resuspended cells. The cells were then disrupted using a BioSpecs Products minibead-beater for 8 rounds of 30 sec pulse and 30 sec rest. After bead-beating, the supernatant was transferred to a new microfuge tube and centrifuged to remove cell debris and beads.

2.6.2 Sonication

5 ml of culture was harvested by centrifuging at 13,000 rpm, 30 sec. Then the cells were washed with 1ml of lysis buffer and harvested again. The supernatant was discarded, while the cells were resuspended in 500 µl sonication buffer. The cell mixture was then sonicated in 30 sec pulse and 30 sec rest on ice for 6 rounds (the Microson™ ultrasonic cell disruptor was set 13,000 – 14,000 watt). The ultrasonic probe was cleaned with 70% ethanol for every new run. After sonication, the supernatant was transferred to a new microfuge tube and centrifuged to remove cell debris.

2.6.3 Bradford assay

Bradford assays were done to measure the protein concentration in the samples which were then used to normalize values from enzyme assays. 1 ml of cell culture was harvested by

centrifuging at 13,000 rpm, 1 min and then re-suspended in 500 µl of 0.25M NaOH. The tube was incubated in a heat block at 100ºC for 5 min. 500 µl of 0.5M HCl was added to the tube followed by vortexing. The sample was subsequently centrifuged at 13,000 rpm for 1 min at 4°C. 10 µl of supernatant was removed and added to 90 µl of Bradford reagent. The mixture was mixed well before the absorbance at 595 nm was taken. The standard curve was also constructed every time that this assay was carried out by mixing 10 µl of standard BSA of various concentrations (0, 50, 100, 200, 300, 400, 600, 800 and 1000 µg/ml) with 90 µl of Bradford reagent and measuring the absorbance at 595 nm.

2.6.4 SDS PAGE and His-tag staining

To detect the expression of His-tagged proteins, *S. meliloti* strains were subcultured in TY at 30°C starting at OD_{600} of 0.1, followed by adding IPTG to 0.4 mM when OD_{600} reached 0.4 and incubating at room temperature overnight for expression. Approximately 5 ml of overnight culture was harvested by centrifuging at 17,000 g for 1 min. The pellet was resuspended in 10 µl loading dye and 40 µl distilled water before incubation at 95°C for 5 min to lyse the cells. The mixture was then centrifuged at 17,000 g for 1 min to remove cell debris. Then 10 µl of sample was loaded onto a 8% polyacrylamide gel and run at 200 V for 1 h using the Bio-Rad Power Pac 3000 apparatus. Finally, the gel was used for His-tag staining following the InVision™ His-tag In-Gel Stain protocol provided by Invitrogen.

2.7 Polymer-related work

2.7.1 Gas chromatography (GC)

Intracellular polymer production was evaluated by gas chromatography following a protocol which has been described by others (Braunegg et al., 1978; Jung et al., 2009). Briefly, cells were harvested from flask culture after a 3-day incubation by centrifuging at 4,000 g for 20

min, then washed twice with distilled water, and finally dried at 100°C overnight. The dried cell weight (DCW) was recorded before methanolysis in 2 ml chloroform and 1 ml PHA solution containing 8 g benzoic acid l^{-1} as an internal standard and either 30% sulfuric acid (for SCL PHA, PLA or P(3HB-*co*-LA) analysis) or 15% sulfuric acid (for MCL PHA) in methanol. The reaction was carried out at 96°C for 6 h, cooled, and then 1 ml of water was added, the mixture was vortexed, and the solution was allowed to separate into two phases. 1 µl of the chloroform phase was taken for analysis by GC as previously described (Jung et al., 2009). The samples were injected into Agilent 6890 series GC system with a DB Wax column (30 m x 0.53 mm, film thickness 1 µM, J & W Scientifics), which is located in the Department of Chemical Engineering. The oven program was set as following: initial temperature was set at 80ºC for 5 min, then ramped to 230ºC at 7.5ºC/min, and continued to ramp to 260ºC at a faster rate 10ºC/min followed by maintaining that temperature for 5 min.

2.7.2 Gas chromatography/ Mass spectrometry (GC/MS)

The samples analyzed using GC/MS were prepared similar to those analyzed using GC as described earlier. The samples were injected into Agilent Technologies 6890N Network GC system with MS detector (inert XL EI/CI MSD 5975B, Agilent Technologies) and capillary HP-5MS 5% Phenyl Methyl Siloxane column (30m x 0.25 mm, film thickness 0.25 µM, Agilent 19091S-433) located in the Department of Chemistry . The oven program was set as following: initial temperature was set 50ºC for 5 min, then ramped to 280ºC at 20ºC/min, and held for 10 min.

2.7.3 Nuclear magnetic Resonance (NMR)

NMR experiments were performed using Bruker 500MHz UltraShield^{TM} spectrometer following the protocol as described previously (Jung et al., 2010b). A proton with 16 scans was

run and integrated to show how much H there was (for example CH2 should integrate to 2 protons). Then a gs-COSY (COrrelation SpectroscopY) was performed to see which protons are coupled to each other, using gradient pulses. The next experiment was HMQC (Hetronuclear Multiple Quantum Correlation) which indicated which protons are attached to which carbons. There must be C-H connection to observe this. Also, HMBC (Hetronuclear Multiple Bond Correlation) was carried out to show the long range H,C correlations via 2 J(C-H) and 3J(C-H) i.e.,2; to 3 bond coupling. This method suppresses the 1J C-H coupling and shows how carbons with no protons are connected. Finally, 3 carbon experiments were run; including 2 dept experiments (dept 135 showed which carbons are CH_3, CH_2, CH, and the dept 90 indicates which carbon is CH only) and a standard experiment that reveals where the carbons are in ppm and indicated all the Carbons with and without protons attached.

2.7.4 Polymer extraction

1 L of culture, cultivated in YEM media for 3 days, was harvested and washed twice with de-ionized water. The cell pellet was subsequently freeze-dried for 6 h. After being freeze-dried, the polymer was extracted in 10 ml chloroform using reflux apparatus for 4 h. Next, cell debris was removed by passing through a filter (number 1 Whatman). Eventually, the polymer was precipitated using 100 ml cold methanol, and the solvent was evaporated by drying in a fumehood overnight. This process was repeated to extract any leftover polymer in the cells. The process was performed as shown in Figure 2.1.

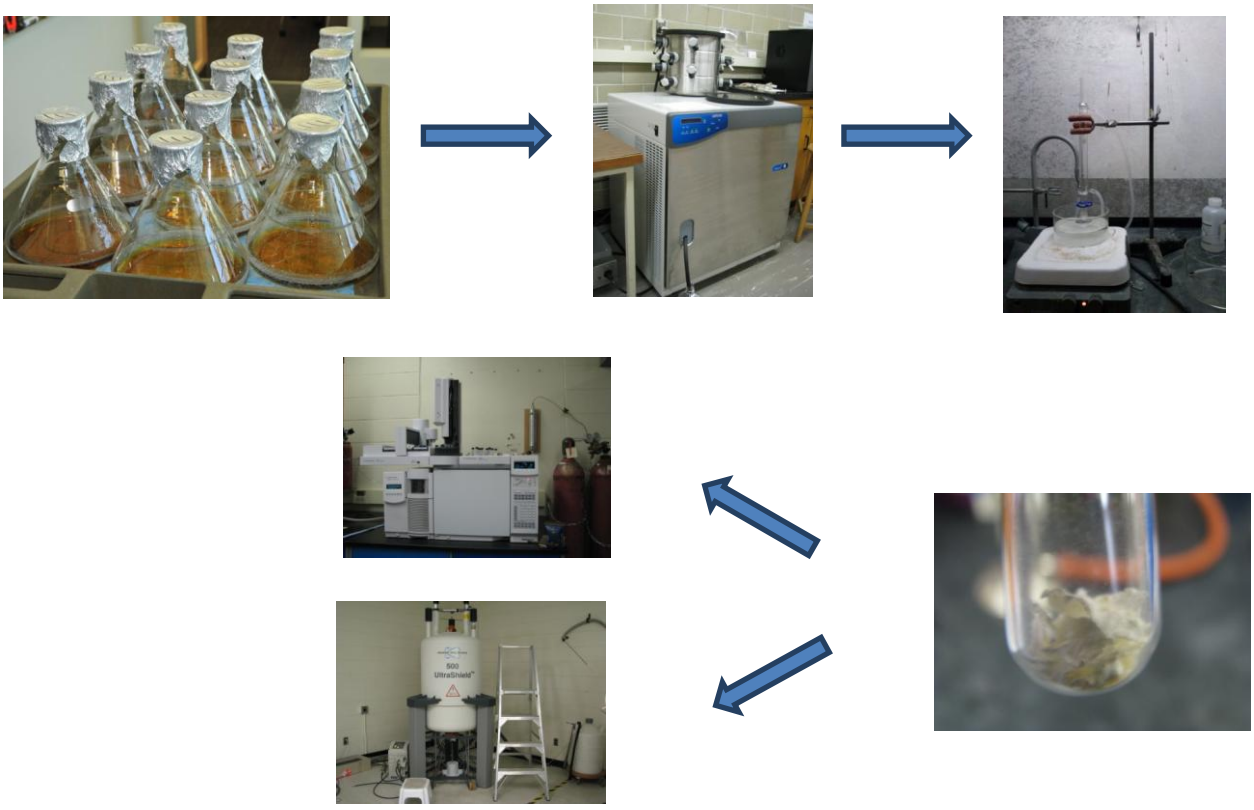


Figure 2.1 Polymer purification and analysis process.

Chapter 3 Chromosome-engineering in *Sinorhizobium meliloti*

3.1 Introduction

A wide variety of biodegradable PHA intracellular polymers are naturally produced in a number of bacteria (Doi et al., 1992; Doi et al., 1995; Hrabak, 1992; Timm et al., 1994; Trainer and Charles, 2006). These polymers vary in their physical properties. Their natural role is typically to cope with the stress associated with fluctuating nutrient availability (Lee, 1996). Several of these polymers have been candidates for use as bioproducts, in many cases to replace fossil fuel-based materials. There are key challenges to the economic viability of production of these biopolymers, especially in competition with the established fossil fuel-based products. Ability to incorporate inexpensive feedstock would improve the economic feasibility of biopolymer production. In addition to naturally produced polymers, there have been efforts to produce new polymers with potentially interesting and useful properties.

PLA is a biodegradable polymer that is currently produced chemically from biologically produced lactic acid derivatives using heavy metal catalysis (Taguchi et al., 2008), but is not known to be naturally produced by any microbe. It possesses several favourable properties such as high strength, biocompatibility, heat resistance, permeability and optical activity that make it a leading candidate for medical applications such as resorbable sutures, drug carriers, organ-substituting implants and artificial blood vessels (Lunt, 1998; Södergård and Stolt, 2002). The co-polymer PLA-*co*-PHA has been obtained through ring-opening polymerization of L-lactide combined with PHA using stannous octoate as a catalyst (Haynes et al., 2007). In part to avoid

contamination with trace levels of heavy metal, research has focused on producing PLA and similar types of polymers through direct fermentation (Jung et al., 2010b; Selmer et al., 2002; Yang et al., 2010), despite the absence of natural production. It was determined that lactic acid (LA) and 3-hydroxyalkanoate (3HA) monomer, especially D-3-hydroxybutyrate, share a similar structure including both hydroxyl and carboxyl groups essential for the formation of an ester bond (Yang et al., 2010).

Because the substrate specificity of PHA synthase enzymes varies, both natural isolates and engineered strains have been shown to successfully produce several different types of PHA copolymers containing both SCL and MCL monomers (Jung et al., 2010a). Based on these observations, efforts have been undertaken to develop a PHA synthase to promote the polymerization of LA-CoA to produce PLA homopolymer or P(LA-*co*-HB) copolymer. Following the reported discovery of a diverse range of PHA synthase substrates by an *in vitro* polymerization screening system (Tajima et al., 2004), a number of studies on producing this novel copolymer *in vivo* were undertaken. This resulted in successful *in vivo* production of PLA homopolymer and P(LA-*co*-HA) copolymer (Taguchi et al., 2008). In this study, however, the LA fraction that was incorporated into copolymer was quite low, at approximately 6 mol%. Since then, a number of engineered PHA synthases derived by site-directed mutagenesis as well as saturation mutagenesis have been screened for efficient incorporation of LA-CoA into P(LA-*co*-HA) copolymer (Yang et al., 2010). The best performers were able to direct incorporation of LA monomer up to 50 mol%. The mole fraction of LA monomer was inversely proportional to the molecular weight and the crystallinity of P(3HB-*co*-LA), and directly proportional to the glass transition temperature (Tg) of the polymer. While Tg was apparently affected by the LA

fraction, the melting temperature (Tm) was just slightly affected by the LA monomer composition.

To increase the LA monomer content in the copolymers, not only PHA synthase activity but also the flow of LA-CoA precursors needs to be considered. Efforts to construct a more efficient strain, with higher flux of LA-CoA precursors, were first carried out on an *E. coli* model system that was designed to produce either PLA or P(LA-*co*-HA). The first strategy aimed at engineering the propionate CoA transferase enzyme from *Clostridium propionicum,* which converts lactate into lactyl-CoA, by site-directed mutagenesis as well as saturation mutagenesis to improve the specificity of the enzyme (Yang et al., 2010). Subsequent strategies focused on modifying *E. coli* genes to either up-regulate the enzyme (D-lactate dehydrogenase *ldhA,* acetyl-CoA synthetase *acs*) or abolish the enzyme (acetate kinase *ackA,* phosphoenolpyruvate carboxylase *ppc*, and acetaldehyde/alcohol dehydrogenase *adhE*) in order to draw LA monomer precursors toward the polymerizing step (Jung et al., 2010b).

Given the goal of more efficient and less expensive production of LA-containing polymer, we decided to investigate the use of *Sinorhizobium meliloti* as a production platform. This soil bacterium, best known for its N_2-fixing symbiosis with *Medicago sativa* (Alfalfa), is genetically amenable and metabolically versatile (Bélanger et al., 2009a; Gurich and González, 2009). This organism has the advantage of having been used for many years in the agricultural inoculant industry, culturable to very high titres. It is a native P(3HB) producer that can accumulate P(3HB) up to 60-80% cell dry weight under growth-limiting conditions in the presence of excess carbon source (Trainer and Charles, 2006). The P(3HB) cycle in *S. meliloti*, which includes the well-studied P(3HB) synthesis and degradation pathways, resembles the P(3HB) cycles in other native P(3HB) producers. The first step in this cycle is the conversion of

acetyl-CoA into acetoacetyl-CoA catalyzed by *phbA*-encoded ketothiolase (EC 2.3.1.9). This is followed by acetoacetyl-CoA reductase (EC 1.1.1.36) encoded by *phbB* to convert acetoacetyl-CoA into 3-hydroxylbutyryl-CoA. In the last step of the P(3HB) synthesis pathway, *phbC*-encoded P(3HB) synthase (EC 2.3.1.B2) is responsible for polymerizing 3-hydroxylbutyryl-CoA into PHB. In *S. meliloti,* other enzymes taking part in this pathway are P(3HB) depolymerase (*phaZ*) (EC 3.1.1.75), 3-hydroxybutyrate dehydrogenase (*bdhA*) (EC 1.1.1.30), and acetoacetyl-CoA synthetase (*acsA2*) (EC 6.2.1.16).

In this study, we describe a system that enables engineered enzymes, which have been studied previously, to function in *S. meliloti* to produce P(3HB-*co*-LA). The engineered propionate CoA transferase enzyme is required to convert lactate into lactyl-CoA, which can then be polymerized with 3HB-CoA to make P(3HB-*co*-LA) by a modified PHA polymerase (Figure 3.1). The genes for these enzymes have been modified and codon-optimized for expression in *S. meliloti.* They were expressed from the native *phaC* promoter following integration into the *S. meliloti* chromosome.

The advantages of this strategy are that the system is extremely stable and the genes are expressed using the native promoter. The genes are introduced into the genome at the *phaC* locus, hence they will be maintained permanently without the need of using antibiotics. We also took advantage of native promoter to express synthesized genes.

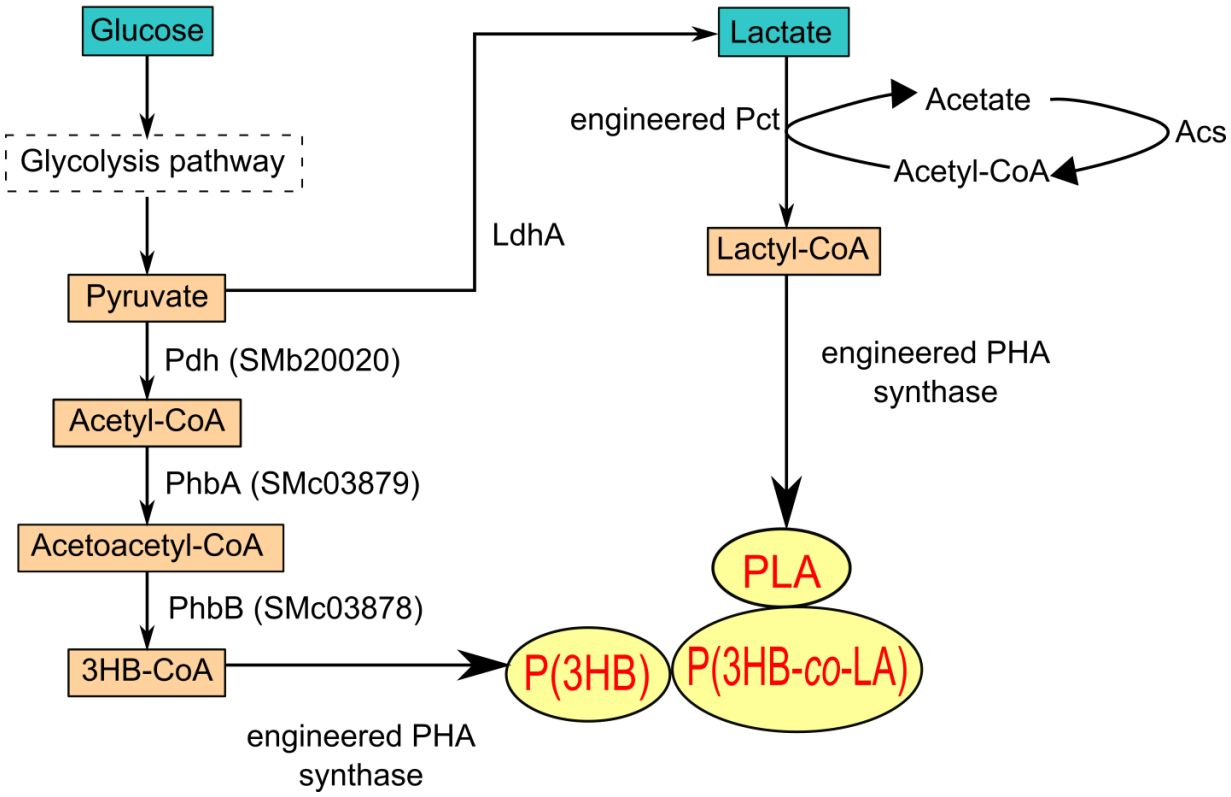


Figure 3.1 Metabolic engineering in *S. meliloti* for the production of novel polymers

3.2 Results and Discussion

3.2.1 The rationale of employing codon-optimized engineered genes

The amino acid sequences of two engineered genes *pct532* and *phaC1400*, which encode engineered propionate CoA transferase from *C. propionicum* DSM 1682 and engineered PHA synthase I from *Pseudomonas* sp. MBEL 6-19, respectively, were used; *pct532* had been randomly mutated using error-prone PCR (A243T, one silent nucleotide mutation of A1200G), while gene *phaC1400* was engineered by saturation and site-directed mutagenesis (E130D, S325T, S477R, Q481M). Combining these evolved genes has been proved to improve the lactate fraction in P(3HB-*co*-LA) copolymer production (Jung et al., 2010b). These genes were codon-optimized for *S. meliloti* using the codon adaptation tool JCat (Grote et al., 2005). The optimized sequences, with a His tag of six histidine residues at the end of each gene (before the stop codon), and a ribosome binding site (RBS) at the beginning of each gene, were then synthesized on a single fragment, with *phaC1400* immediately following *pct532*. The DNA sequence of the synthesized fragment has been included in Supplementary 1.

Comparing the engineered genes before and after codon optimization revealed that the overall GC content increased for both genes (for the *pct* gene, the GC content increased from 40.38% to 53.65%; for the *phaC* gene, the GC content increased from 58.86% to 62.14%), while the codon diversity decreased across both genes (for the *pct* gene, the number of codons decreased from 52 to 37; for the *phaC* gene, the number of codons decreased from 56 to 39). The *pct* gene originated from *Clostridium propionicum* which has fairly low GC content compared to *S. meliloti*. Therefore, there was a significant increase in GC content for this gene. After the codons were optimized, there were four scenarios observed: the appearance of new codons, the disappearance of existing codons, increase of the proportion of certain codons, and the

maintenance of the same percentage of others. Meanwhile, the *phaC* gene originated from *P. putida* had GC content that is quite similar to that of *S. meliloti*. This explains why the GC content of this gene remained nearly the same. There were also four changes in the replacement of codons in *phaC* gene, but not all the same. The first scenario which is the appearance of new codons was not found in this optimized gene. Instead, the remarkable decrease of only one codon (CAA encoding glutamine) was seen. However, these genes were only partially optimized since we also desire to examine these genes in a broad range of different host backgrounds which have different codon usage and prefer lower GC content. Had we not chosen "the partial optimization" option, the GC content would have increased more (64.7% and 65.11% for the *pct* and *phaC* genes, respectively); and the number of codons would have severely dropped to 20 codons for both of the genes. These fully optimized sequences could be the ideal sequences for expression in *S. meliloti*, but this also would restrict the genes from being expressed in other host strains. Hence, there is a trade-off between gene expression optimization and host strain flexibility.

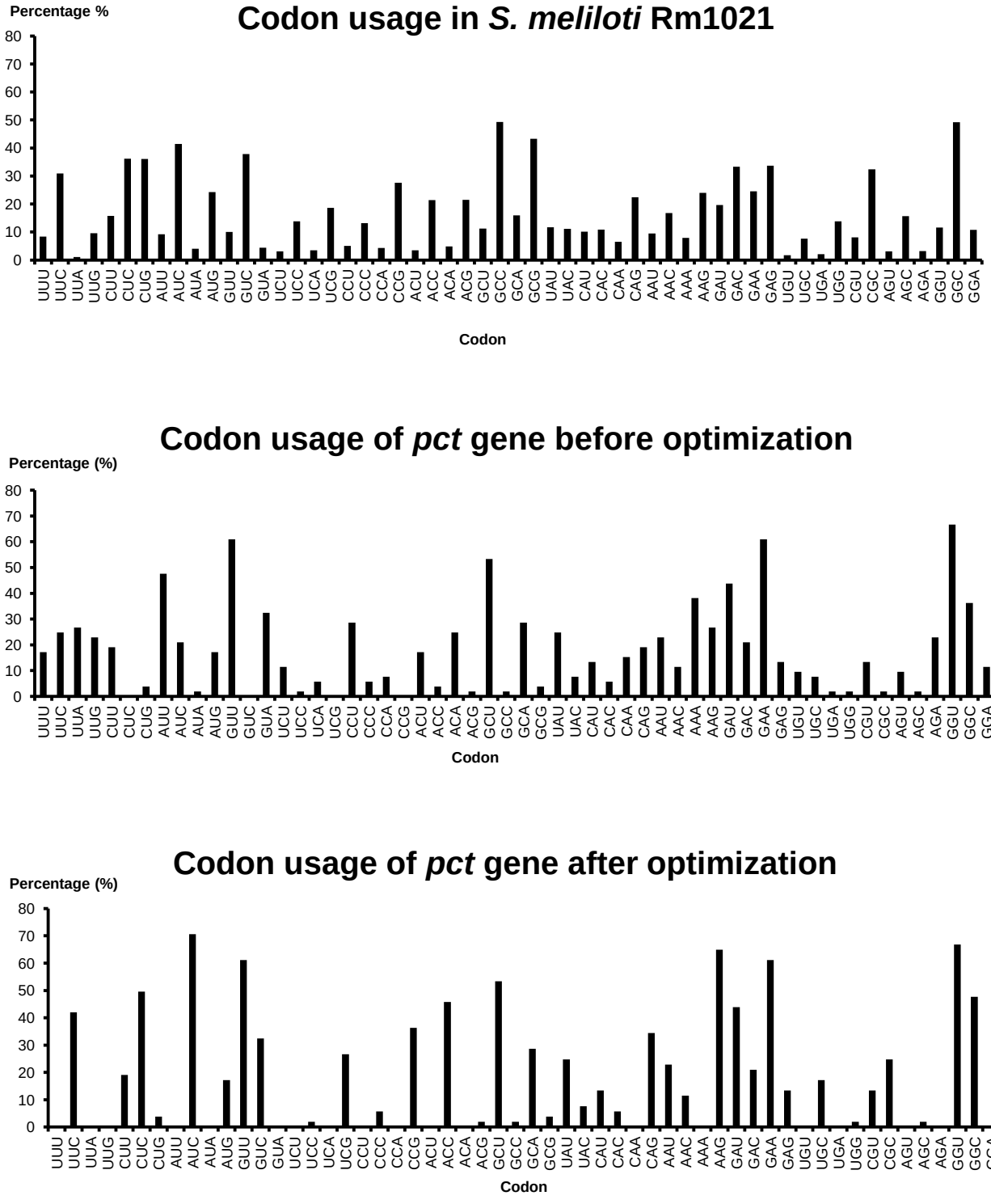


Figure 3.2 Comparison of codon usage *in S. meliloti*, codon usage of engineered *pct* gene before and after optimization

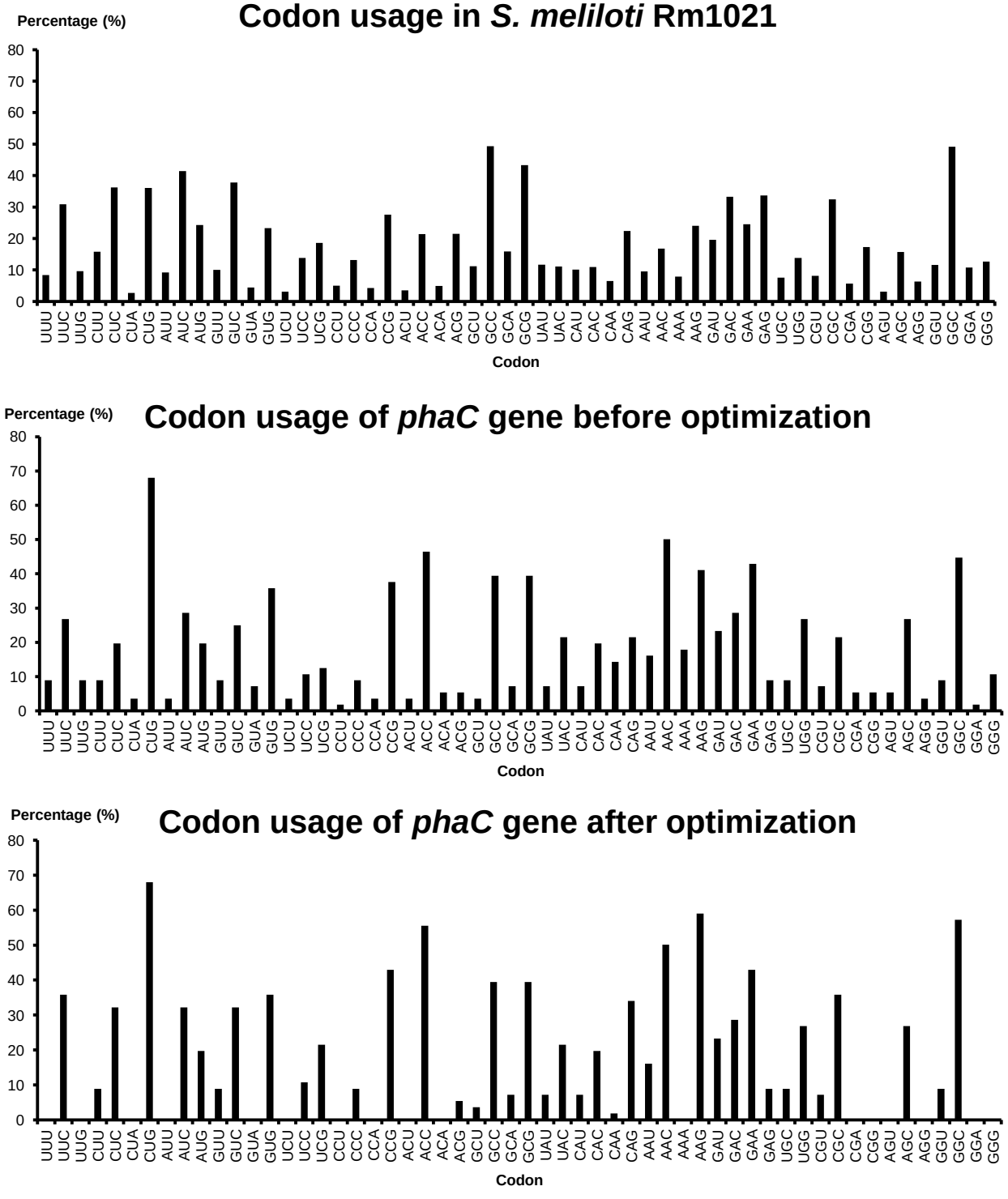


Figure 3.3 Comparison of codon usage in *S. meliloti*, codon usage of engineered *phaC* gene before and after optimization

3.2.2 Construction of S. meliloti strain harbouring synthetic codon-optimized *pct532* and *phaC1400* genes integrated into the chromosome

We constructed a suicide plasmid for substitution of the *S. meliloti phaC* gene, which encodes the native PHA synthase enzyme, with the codon optimized *pct532* and *phaC140* genes, under the control of the native *phaC* promoter. To achieve this, roughly 300 bp-long fragments were taken from immediately upstream of the *S. meliloti phaC* start codon and immediately downstream of the stop codon; the fragments were designed to flank the optimized *pct532* and *phaC140* genes, and this designed construct was chemically synthesized (Biobasic). The construct was then cloned into the *Xba*I site of the suicide vector pK19*mobsacB* to create pK19*pctPhaC* as shown in Figure 3.4. This plasmid was introduced into *S. meliloti* Rm11144, which harbours a Tn5-233 insertion in *phaC* (Trainer et al., 2010), by triparental conjugation from *E. coli* DH5α, with selection for Nm^r. Double crossover mutants were recovered following selection on TY containing 5% sucrose, followed by screening for loss of Tn5-233-encoded Gm/Sp^r. This process is illustrated in Figure 3.5 .Two colonies, which were neither sucrose-sensitive nor Gm-sensitive, were randomly picked to be tested by colony PCR. Colony PCR analysis with two sets of primers (VPhbC_F and VPhbC_R1; VPhbC_F1 and VPhbC_R) confirmed precise replacement of the *phaC* ORF by the synthesized genes. The resulting strain was designated SmUW254. The results were shown in Figure 3.6. Primers VPhbC_F and VPhbC_R1 were designed to be complementary to a region outside homologous region 1 (HR1) and within the *pct* gene, respectively. Primers VPhbC_F1 and VPhbC_R were designed to be complementary within the *phaC* gene and outside homologous region 2 (HR2). Lane 1 and lane 2 showed expected bands (1194 bp and 1116bp, respectively) using above primer sets to confirm

the integrant strain SmUW254. Lane 3 and lane 4 did not show any bands using the same primer sets in the parental strain Rm11144.

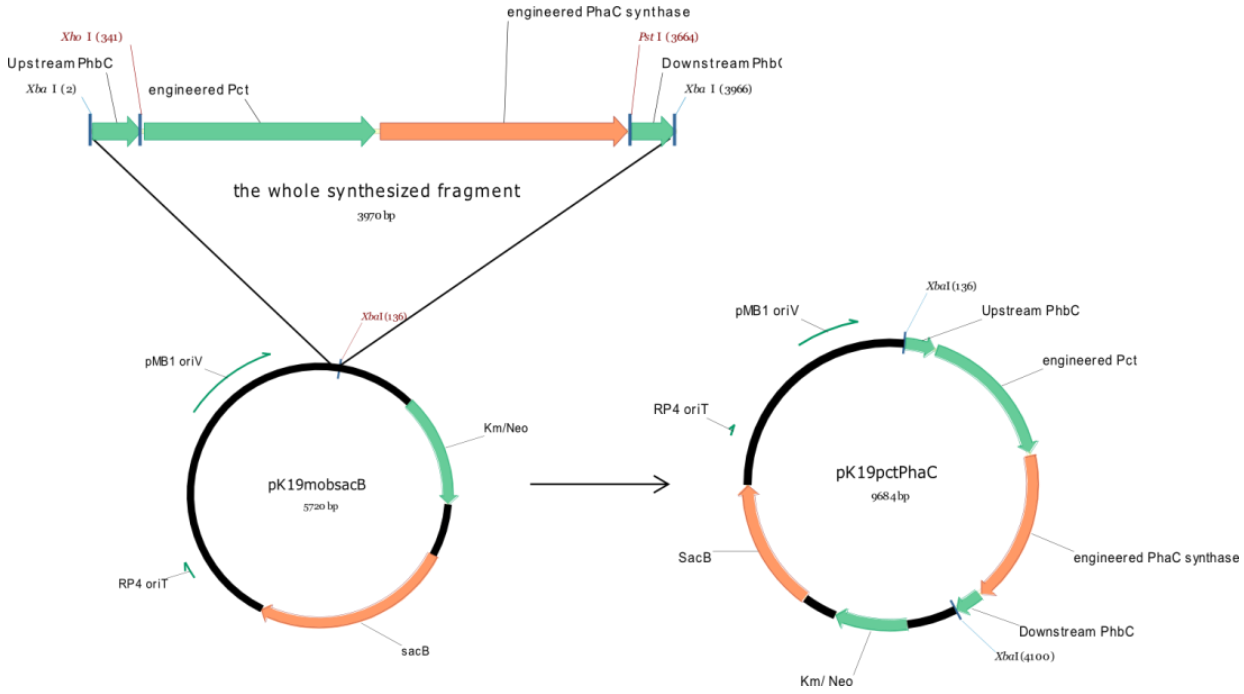


Figure 3.4 Plasmid construction of suicide vector pK19*pctphaC*. The synthesized fragment, including two synthesized genes (*pct* and *phaC*) flanked by upstream and downstream phbC fragments, was cloned into pK19*mobsacB* to create suicide vector pK19*pctphaC*.

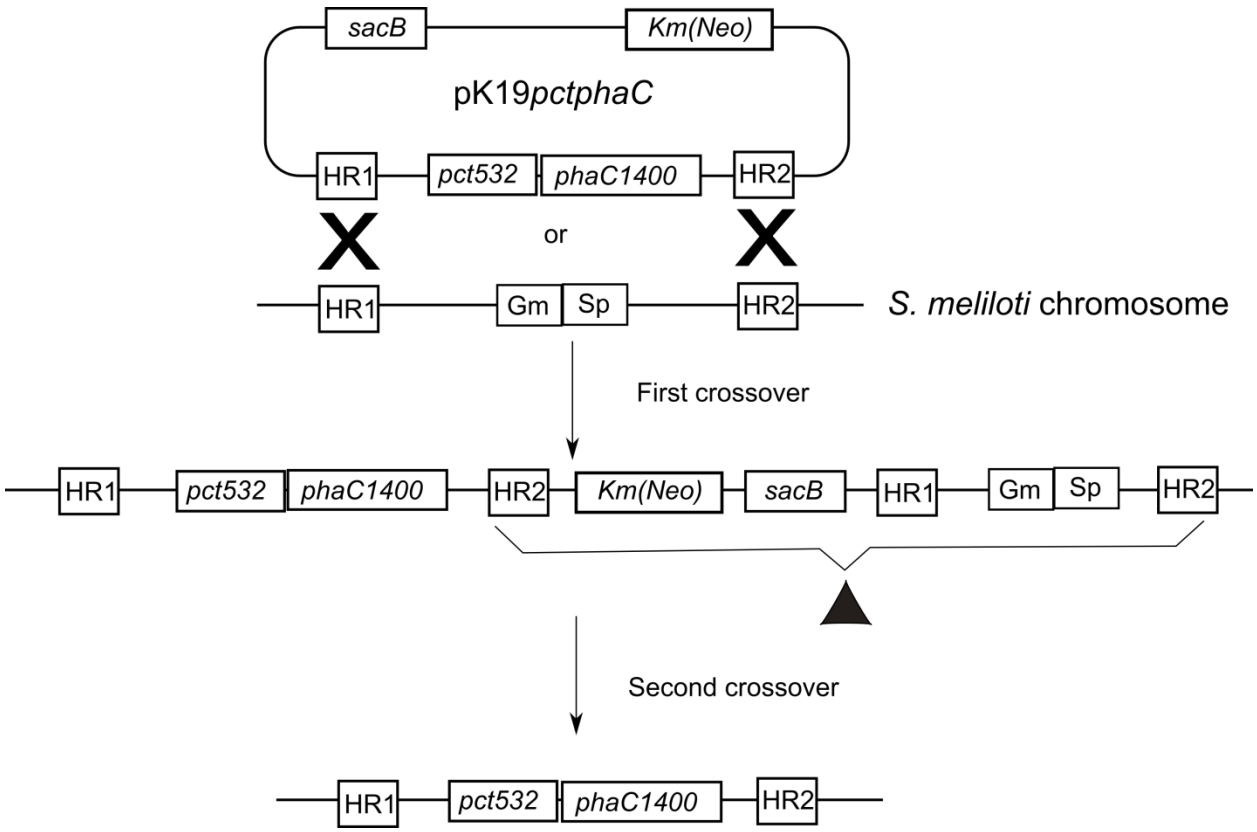
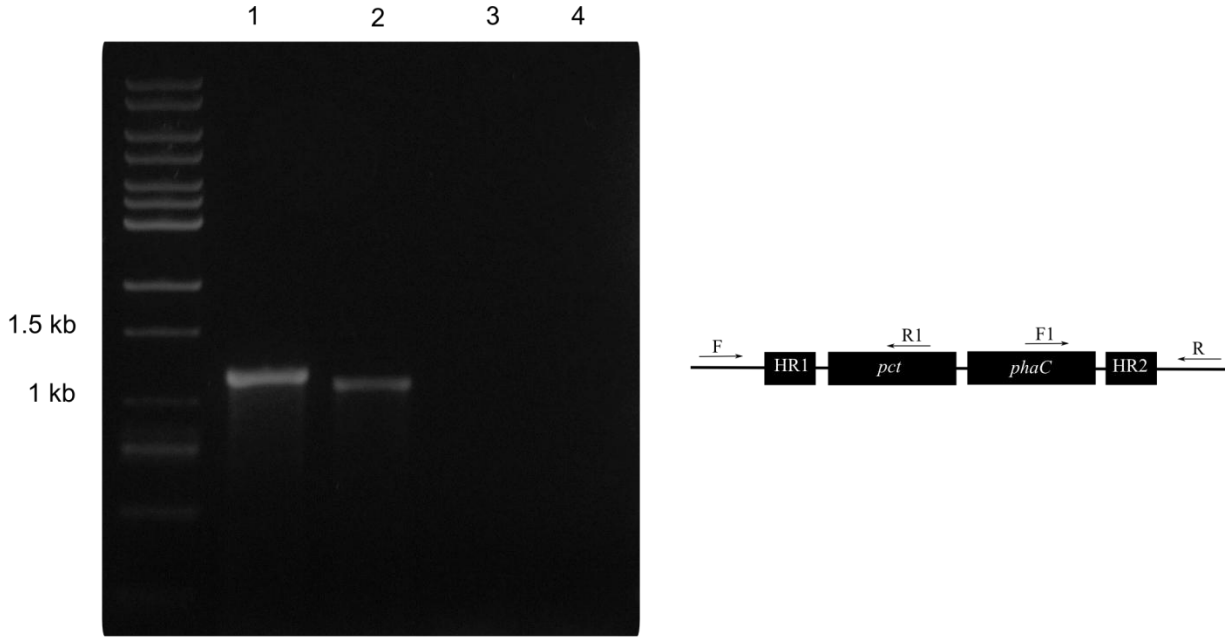


Figure 3.5 Diagram of gene integration into *S. meliloti* background using a double-crossover technique.



1 integrant strain SmUW254 (primers F and R1, 1194bp)
2 integrant strain SmUW254 (primers F1 and R, 1116bp)
3 parental strain Rm1144 (primers F and R1)
4 parental strain Rm1144 (primers F1 and R)

Figure 3.6 Confirmation of a successfully gene-integrated strain using colony PCR

3.2.3 Phenotypic analysis of strain constructs

Initial confirmation of polymer production in the engineered strains was performed on Nile Red-containing YM agar plates using the Fluorescence Theatre (Heil et al., 2011). The strain SmUW254 were streaked out alongside Rm1021 and Rm11144 as *phaC*-positive and *phaC*-negative controls, respectively (Figure 4). SmUW254 exhibited fluorescence similar to the wild-type strain Rm1021, while the *phaC* mutant strain Rm11144, which cannot produce P(3HB), was deficient in fluorescence. The engineered strains also restored the exopolysaccharide-linked mucoid colony morphology on high-carbon growth media that is correlated with PHA accumulation (Aneja et al., 2004; Schallmey et al., 2011).

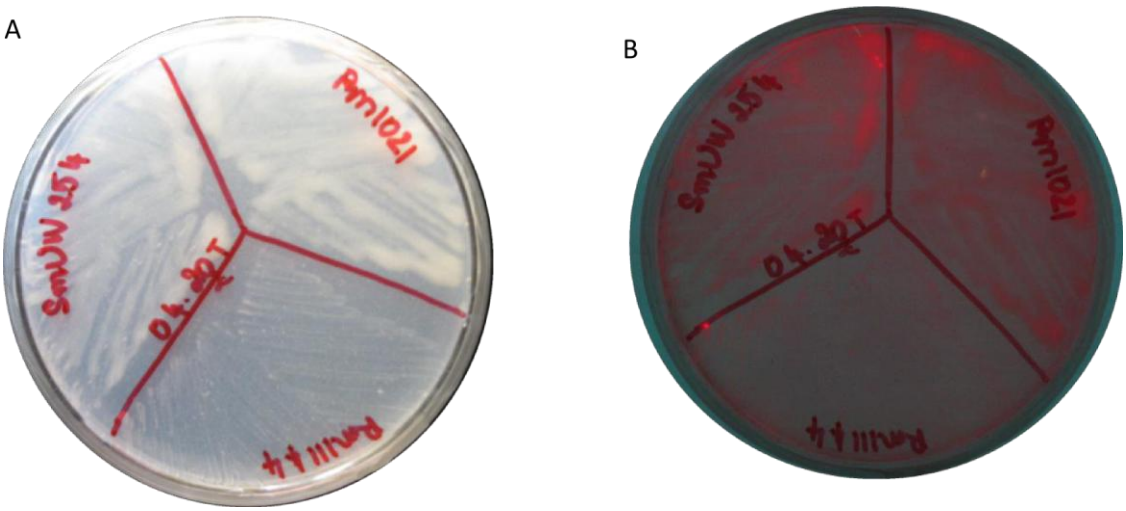


Figure 3.7 Phenotypic complementation of chromosome-engineered strains. A and B demonstrate phenotypic difference between chromosome-engineered *S. meliloti* strains and other *S. meliloti* strains: parental strain Rm1021 (*phbC*+), mutant strain Rm11144 (*phbC*) and engineered strain SmUW254. A) normal image, B) fluorescent image

3.2.4 Polymer production in chromosomal engineering system

The major objective of this study was to engineer *S. meliloti* to produce novel polymers. Integration of the engineered genes onto the chromosome should improve production strain stability. The chromosomal integrant strain SmUW254 can produce polymer content of 15% approximately, which is slightly lower than the wild-type strain (20%). However, LA can only be detected in the engineered strain SmUW254 and constituted 30% mol (Figure 3.8). An important difference between using *S. meliloti* and *E. coli* is that the production of co-polymer in the engineered *S. meliloti* strain did not require the provision of exogenous 3HB substrate, since D-3-hydroxybutyrl-CoA is naturally formed through the action of the native *phaAB* gene products.

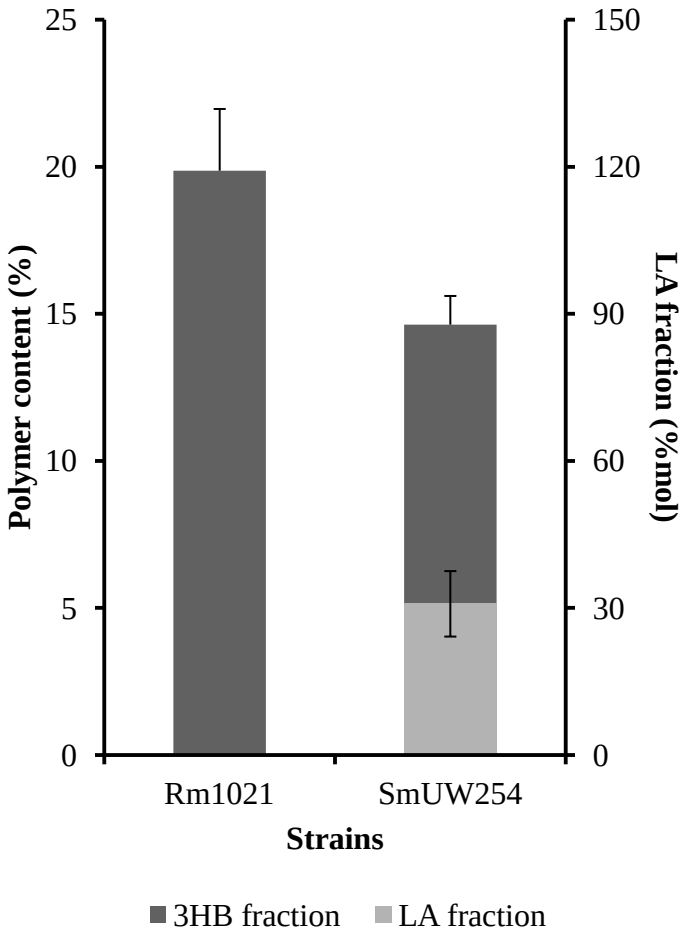


Figure 3.8 Polymer production in engineered strains. Polymer content and LA fraction analysis in parental strain Rm1021 and engineered strain SmUW254. Only SmUW254 can accumulate roughly 30 mol% LA. The experiment was done in duplicate. Error bars represent the range of the mean.

To further investigate as well as confirm the structure of this copolymer, we carried out the purification of the copolymer. The purified polymer was analyzed using GC/MS and NMR. The NMR ^{1}H and ^{13}C spectrum of the purified polymer were identical to those of PLA homopolymer from published studies (Jung et al., 2010b; Taguchi et al., 2008; Yang et al., 2010), except for CH_2 peak which accounted for the presence of 3HB monomer (Figure 3.9). To confirm the presence of LA monomer, we also carried out the analysis of structure using GC/MS. The result also matched the retention time of the LA standard and the mass spectrum in the library (Figure 3.10).

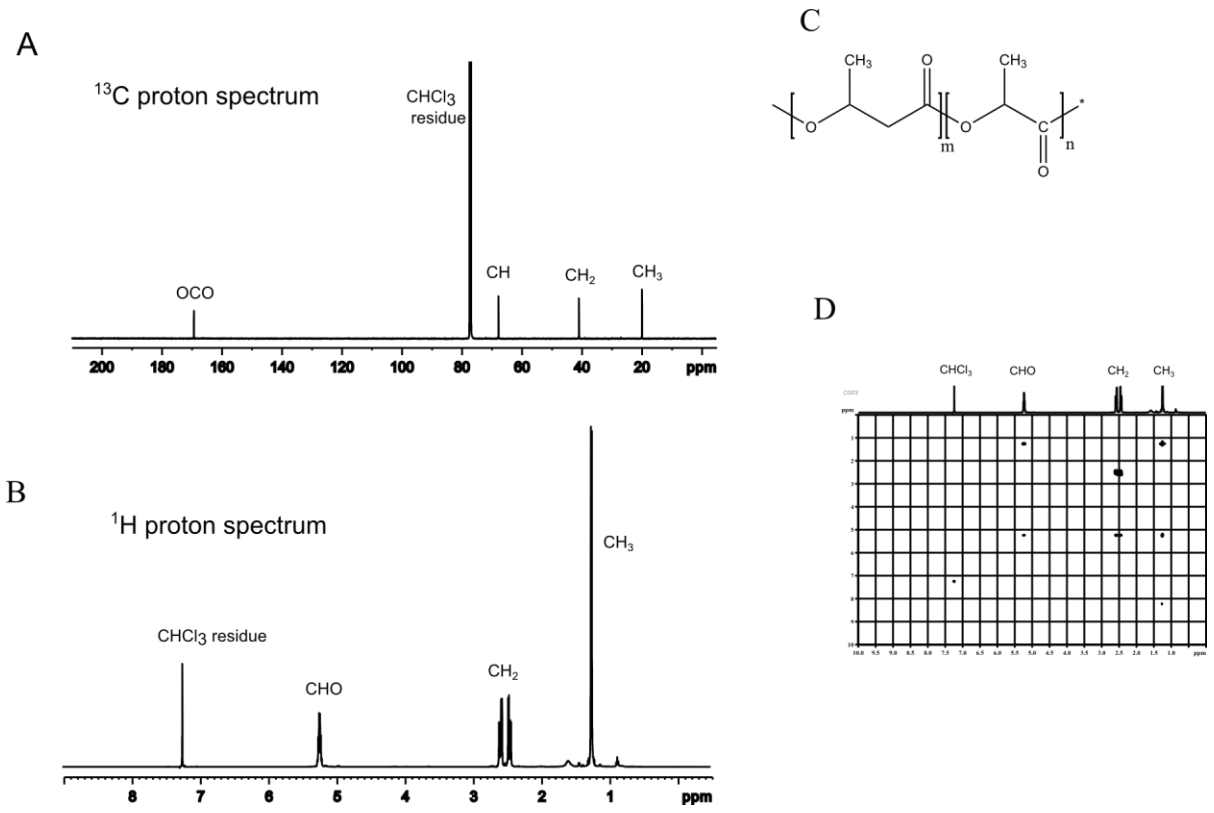


Figure 3.9 NMR analysis of purified polymer. A. The ^{13}C NMR spectrum of purified polymer. B. The ^{1}H NMR spectrum of purified polymer. C. The chemical structure of purified polymer. D. The COSY NMR spectrum of purified polymer.

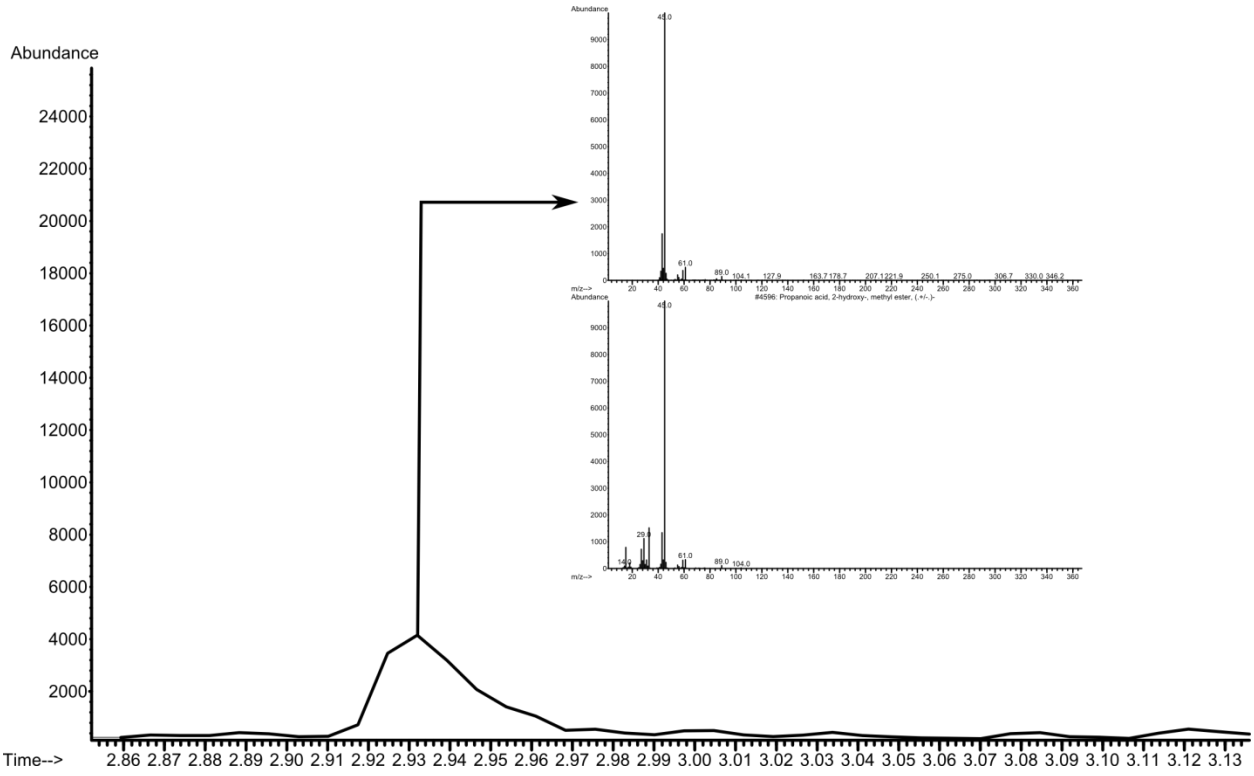


Figure 3.10 GC/MS analysis of purified polymer. The figure shows the LA monomer fraction peak. The arrow indicates the mass spectrum of this fraction (top) which matched with the LA structure in the library (bottom).

3.3 Conclusions

Here we have presented the strategy of chromosome engineering to engineer *S. meliloti* to make it a robust platform for polymer production. It was shown that this system was able to produce P(3HB-*co*-LA) copolymer. Our work has shown for the first time that *S. meliloti* has the ability to produce this type of polymer through the application of metabolic engineering tools. We intend to follow these results with further in-depth studies on biopolymer production in general, and novel polymer production in *S. meliloti* specifically.

Chapter 4 Plasmid-based engineering in *Sinorhizobium meliloti*

4.1 Introduction

In this chapter, another strategy has been used to engineer *S. meliloti* as a host for the production of novel polymers. Engineered genes which have been described in detail in Chapter 3 were expressed from a plasmid rather than from the chromosome. There have been a number of studies which employed this strategy to examine heterologous gene expression in bacteria, commonly *E. coli*, using constitutive or inducible promoters (Borrero-de Acuña et al., 2014; Fukui et al., 1998; Fukui and Doi, 1997; Klinke et al., 1999; Taguchi et al., 2008). For the production of P(LA-*co*-3HB) copolymer or PLA homopolymer, previous studies employed *E. coli* as a host and expressed engineered heterologous genes carried by a plasmid (Jung et al., 2010b; Taguchi et al., 2008; Yang et al., 2010).

The expression vector pTH1227 that was used is a pFUS derivative vector carrying an RK2 origin, inducible pTac- $lacI^q$ promoter and *gusA* gene as a reporter (Cheng et al., 2007; D'Haeze et al., 2004; Marsh et al., 1984; Reeve et al., 1999; Spaink et al., 1987). This vector is a broad-host range expression vector that has been used to study the role of the *minCDE* genes in both *S. meliloti* and *E. coli* (Cheng et al., 2007). The related vector pMP220 has been used in *Azorhizobium caulinodans* (D'Haeze et al., 2004). It was also used for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule bacteria such as *S. meliloti*, *Bradyrhizobium* sp. and *R. leguminosarum* bv. *Viciae (Reeve et al., 1999)*. The *gusA* gene from *E. coli* encoding β-glucuronidase was used in different host backgrounds as a reporter

gene (Cheng et al., 2007; Myronovskyi et al., 2011; Reeve et al., 1999; Russell and Klaenhammer, 2001; Saborido Basconcillo et al., 2009). The β-glucuronidase (Gus) product can be easily detected by observation of colour change on media containing chromogenic substrates such as X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide, cyclohexyl-ammonium salt), other 3-indoxyl derivatives, and naphthol-β-D-glucuronide. In addition, it also can be quantitatively measured by spectrophotometry (p-nitrophenyl β-D-glucuronide and phenolphthalein-β-D-glucuronide), fluorimetry (4-methylumbelliferyl-β-D-glucuronide and 5-dodecanoyl- aminofluorescein-di-β-D-glucuronide) or chemiluminescence (1,2-dioxetane-β-D-glucuronide). Gus has been widely used to study gene expression and other applications because the enzyme is highly stable, resistant and easily detected.

The pTac-*lacI*^{q} transcriptional regulatory system is well known as an inducible system first developed in *E. coli* (Amann and Ptaslme, 1983; De Boer et al., 1983; Letouvet-Pawlak et al., 1990). The *tac* promoter is a hybrid derived from the *lac* and *trp* promoters. It contains the Pribnow box or -10 region of the *lacUV5* promoter with consensus sequence TATAAT and the -35 region of the *trp* promoter with consensus sequence TTGACA at the position -20 with respect to the transcriptional start site. It is a well known strong promoter. The *lacI*^{q} allele of *lacI* has a mutated promoter sequence that results in enhanced expression up to 10-fold (Calos, 1978; Stark, 1987).

The advantages of expressing the gene from a plasmid under the control of a regulatable promoter include flexibility, tunable expression and ability to easily transfer the genes to a broad range of potential host strains.

4.2 Results and discussion

4.2.1 Construction of plasmid expressing synthetic codon optimized *pct532* and *phaC1400* **genes**

The broad host range vector pTH1227, containing the inducible *tac* promoter and *lacI*^{q} along with a downstream *gusA* reporter gene, was digested with *Xho*I and *Pst*I, then ligated with the *Xho*I / *Pst*I -digested synthesized DNA as shown in Fig. 4.1. This resulted in the plasmid construct pTAM. The pTAM plasmid and the empty vector control pTH1227 were separately introduced into *E. coli* DH5α and a selection of *S. meliloti phbC* mutant (SmUW499) strains. Transcription of the introduced genes was confirmed by assay of *gusA*-encoded *β*-glucuronidase activity.

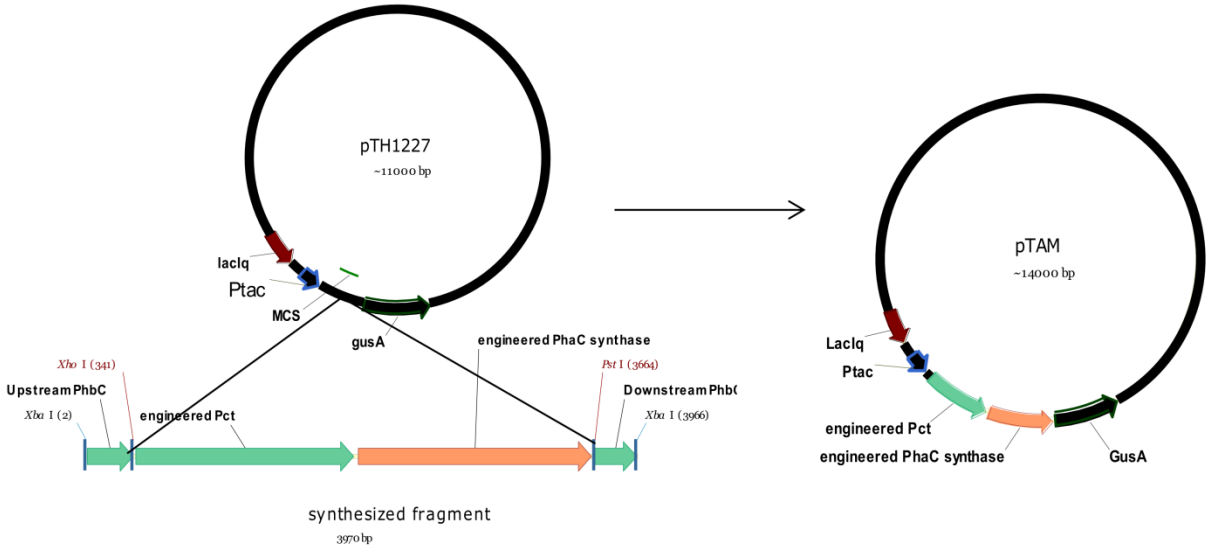


Figure 4.1 Construction of expression plasmid pTAM. Two synthesized, codon optimized genes (*pct* and *phaC*) were inserted as a XhoI-PstI fragment into pTH1227 to create pTAM.

4.2.2 Phenotypic analysis of strain constructs

Initial confirmation of polymer production in the constructed strain SmUW256 was carried out in the same way as previously described for the chromosome engineered strain SmUW254. The strain was streaked out alongside the parental strain and mutant strain as *phaC*-positive and *phaC*-negative controls, respectively. Strain SmUW256, which is the *phaC* mutant containing pTAM, exhibited fluorescence similar to the control strain SmUW257, which contains pTH1227 in a non-mutant *phaC* background. The *phaC* mutant strain SmUW255, containing pTH1227, which cannot produce P(3HB), was deficient in fluorescence, as expected. Interestingly, in the absence of inducer IPTG, we found that SmUW256 exhibited much more fluorescence than with IPTG. This suggested that the gene expression from the plasmid construct was sufficient without IPTG induction to allow complementation.

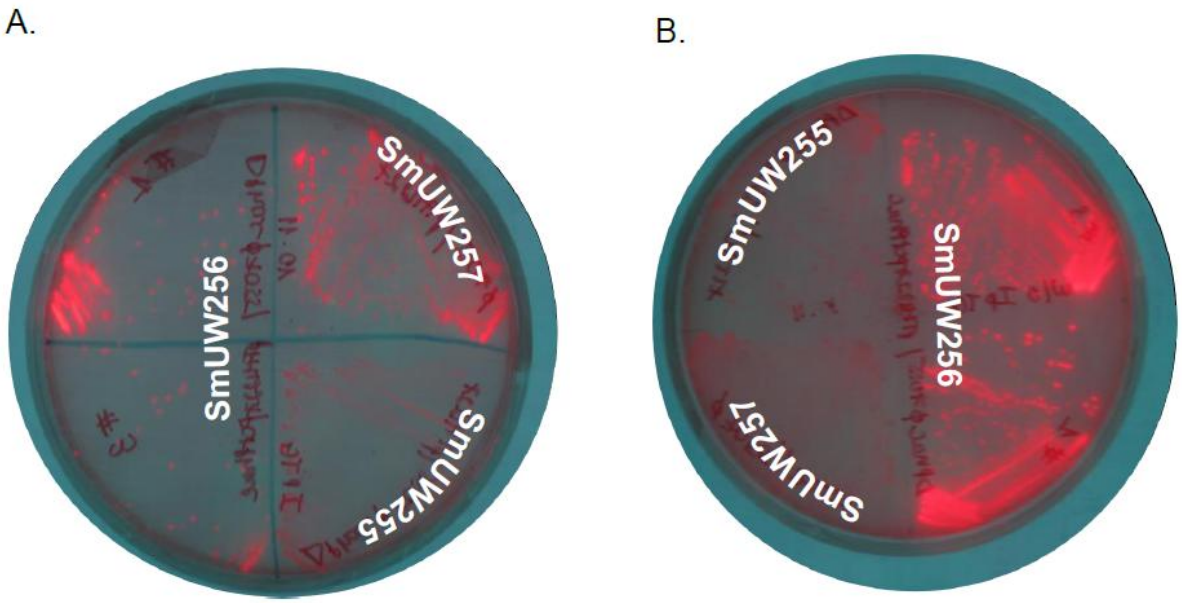


Figure 4.2 Phenotypic complementation of PHA synthesis associated phenotype in *S. meliloti* strains. A and B demonstrate phenotypic difference between between plasmid-based engineered *S. meliloti* strains and other *S. meliloti* strains SmUW255 (*phaC*$^{-}$), SmUW256 (#1, #3), SmUW257 (*phaC*$^{+}$). A) Inducing condition (IPTG), B) Non-inducing condition (No IPTG).

4.2.3 Confirmation of expression of the plasmid-encoded engineered enzymes

To confirm the expression of the engineered enzymes from the pTAM plasmid in the SmUW256 strain, we performed SDS-PAGE and His-tag staining analysis. Each of the two enzymes had a C-terminal 6-His tag. Because the sizes of these two enzymes are very similar (~60 kDa), only one positive band was observed on the gel (Fig. 4.3). We also tested these samples in the absence of IPTG, and surprisingly, a very light band was seen on the gel, suggesting a moderate level of expression under non-inducing conditions. In addition, the function of the engineered enzymes was confirmed by the production of different types of polymers, which were detected by gas chromatography analysis, presented below.

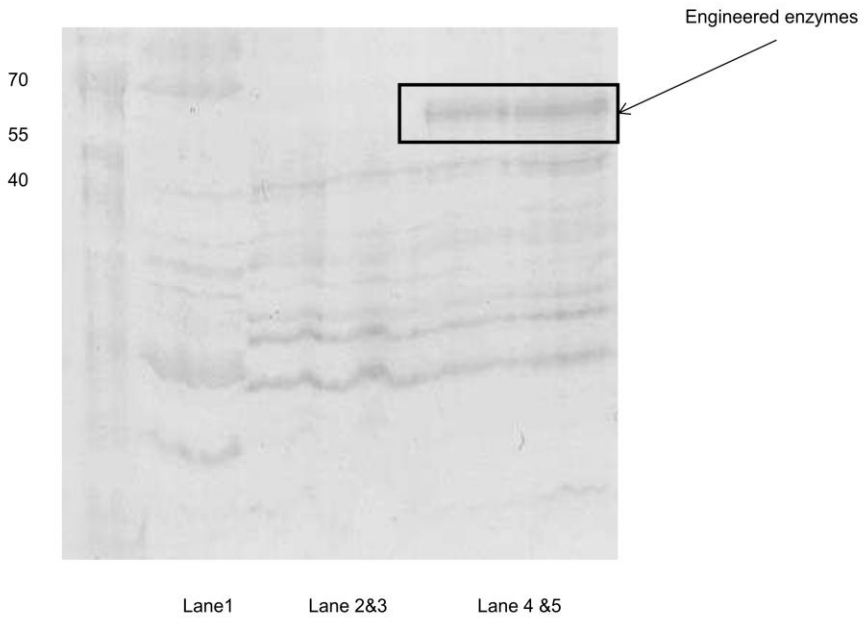


Figure 4.3 Gel image of SDS-PAGE analysis. His-tag staining of positive His-tagged control which is used to test the working conditionof reagents (Lane 1), negative His-tagged control SmUW255 and SmUW257 (Lanes 2 and 3, respectively), and two samples of the plasmid-based construct (Lanes 4 and 5).

4.2.4 Polymer production in plasmid-based system

a. P(3HB) and PLA production

We took advantage of the inducible expression plasmid pTH1227, which has been well-studied in *S. meliloti* (Cheng et al., 2007). In this system, engineered genes were expressed in a *phbC* mutant background under the control of inducible promoter P_{tac} by means of the plasmid-encoded *lacI*^{q}. Surprisingly, in the absence of IPTG supplementation in YM media, levels of P(3HB) produced were similar to the wild-type levels, but in the presence of 0.4 mM IPTG, P(3HB) was only accumulated to about half the wild-type levels. In both conditions, LA was not detected (Figure 4.4). To investigate whether this was due to issues related to competition between P(3HB) and PLA precursors, we blocked the P(3HB) synthesis pathway by using a *phaAB phaC* mutant background. Feeding the strain with lactic acid (10 g l^{-1}) as a mannitol-substituting substrate in YM media resulted in PLA homopolymer production of 4% DCW (Table 4.1), demonstrating that in the absence of competing P(3HB) precursors, the PLA precursors could be incorporated into polymer. Also, we have tested the polymer production in other mutant *S. meliloti* backgrounds (Table 4.2). However, only P(3HB) was found to be produced in these mutants.

b. Investigation of IPTG concentration and induction time

In light of the unexpected observation that greater amounts of polymer were produced in the absence of IPTG induction than in the presence of IPTG, we decided to further investigate the P(3HB) production under different induction conditions. We also measured GusA expression from the downstream *gusA* gene as a proxy for transcription across the engineered genes. A range of IPTG concentrations from 0 to 1 mM was used to induce gene expression (Figure 4.5).

At an IPTG concentration of 0.05 mM, GusA activity in the SmUW256 strain increased markedly compared to that in the absence of IPTG. However, further addition of IPTG did not substantially increase GusA activity in recombinant strain SmUW256. Meanwhile, the control strain SmUW255 which harbours the empty plasmid pTH1227, showed *gusA* expression in rough proportion to IPTG concentration. Contrary to expectations, increased gene expression did not result in more P(3HB) being produced (Fig. 4.7); however, both DCW and yield decreased relative to increased IPTG concentration. Interestingly, the basal level of expression provided the strain with the best conditions to grow and produce P(3HB) based on the highest DCW, yield and P(3HB) percentage. We also tested the ability for P(3HB) production at a range of lower IPTG concentrations (Fig. 4.8). There was no significant difference in P(3HB) accumulation under these conditions, compared to the amount accumulated at the basal level of expression.

Next, the effect of induction timing was investigated to see if the cells behaved differently when 0.4 mM IPTG was added later in the culturing. We observed that the later the IPTG was added, the more GusA activity was decreased (Fig. 4.6). Therefore, if we only consider reporter gene expression level, induction at the beginning of cultivation is the most optimal; however, whether reporter gene expression is directly proportional to P(3HB) production is a separate issue that we sought to resolve. Once again, the results show that this relationship is inversely proportional (Fig. 4.9). Both DCW and yield significantly decreased when the induction occurred at the beginning of cultivation, resulting in the lowest P(3HB) percentage. Induction at Day 1 or 2 of cultivation did not make a significant difference compared to no induction, suggesting that the basal level is still ideal for P(3HB) production.

The question of the optimal level of synthase protein level for polymer production is still unanswered. The rationale is generally that with more protein being expressed, more target

products can be formed. This is why some studies focus on maximizing the products by overexpressing genes in the pathway. However, these two factors are not always directly proportional to each other. High protein levels do not necessarily translate into greater product amounts (Prather, 2010). For instance, it was reported that *E. coli* XL1-Blue harbouring the low-copy-number plasmid pJRDTrcphaCAB$_{Re}$ produced P(3HB) more efficiently than the strain harbouring the high copy number pTrcphaCAB$_{Re}$ (Kahar et al., 2005). However, in this study, the induction time and IPTG concentration were not taken into consideration. In all cases, 0.5 mM IPTG was added into the media at 24 h of cultivation; therefore, it may be that variable results can be obtained using the same low copy number plasmid under different conditions. There is still not a good understanding of the relationship between PHA synthase gene expression and levels of polymer accumulated.

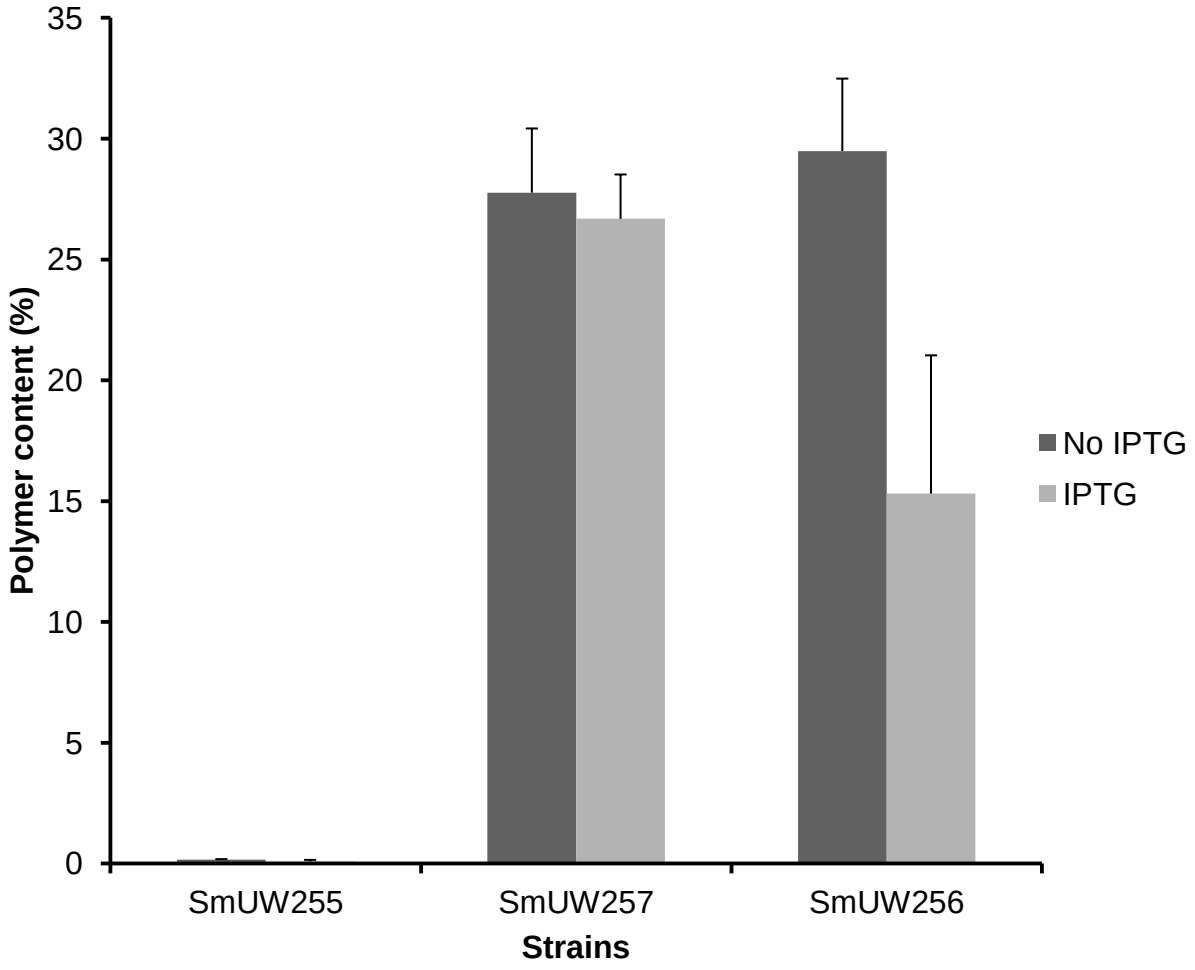


Figure 4.4 Polymer content of SmUW255 (*phaC*$^{-}$), SmUW257 (*phaC*$^{+}$) and SmUW256 (engineered *phaC*). Dark grey bars represent inducing condition (0.4 mM IPTG was added to cultures); light grey bars represent non-inducing condition (no IPTG). The experiment was done in duplicate. Error bars represent the range of the mean.

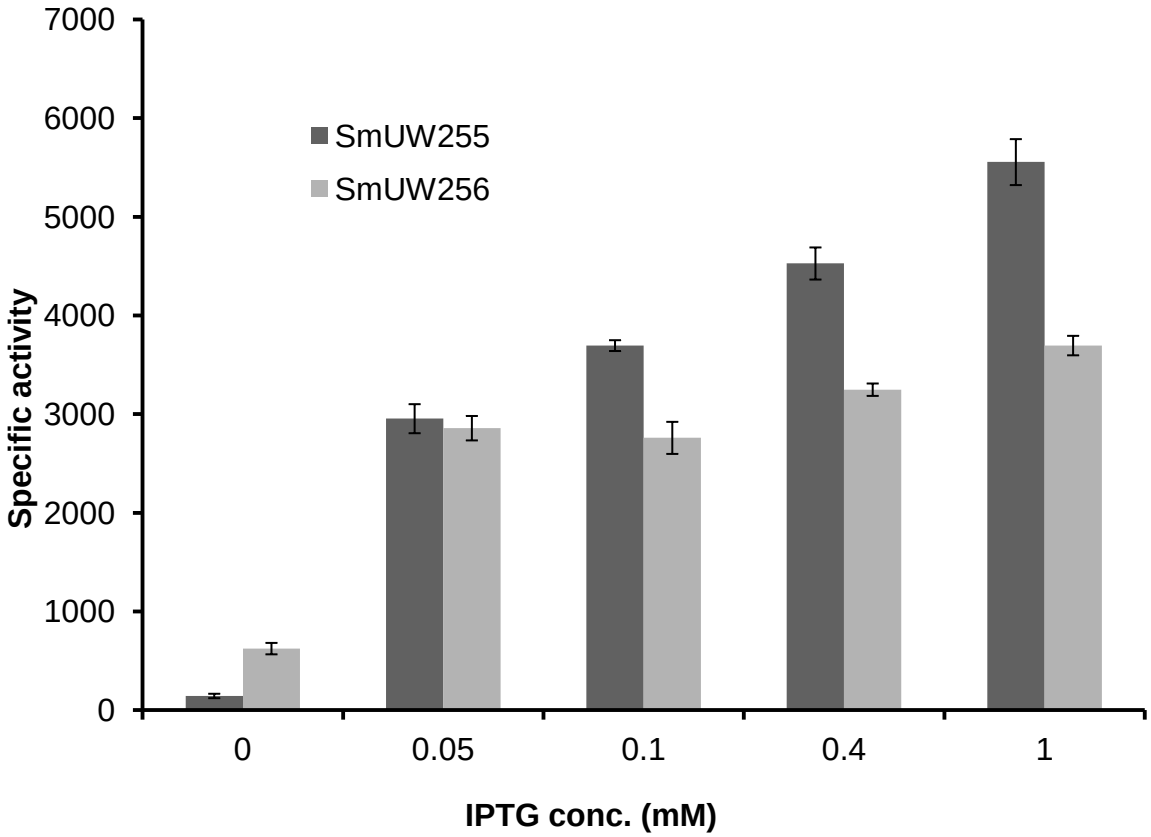


Figure 4.5 Measurement of *gusA* activity of strain SmUW256 induced at different concentrations of IPTG. The experiment was done in duplicate. Error bars represent the range of the mean.

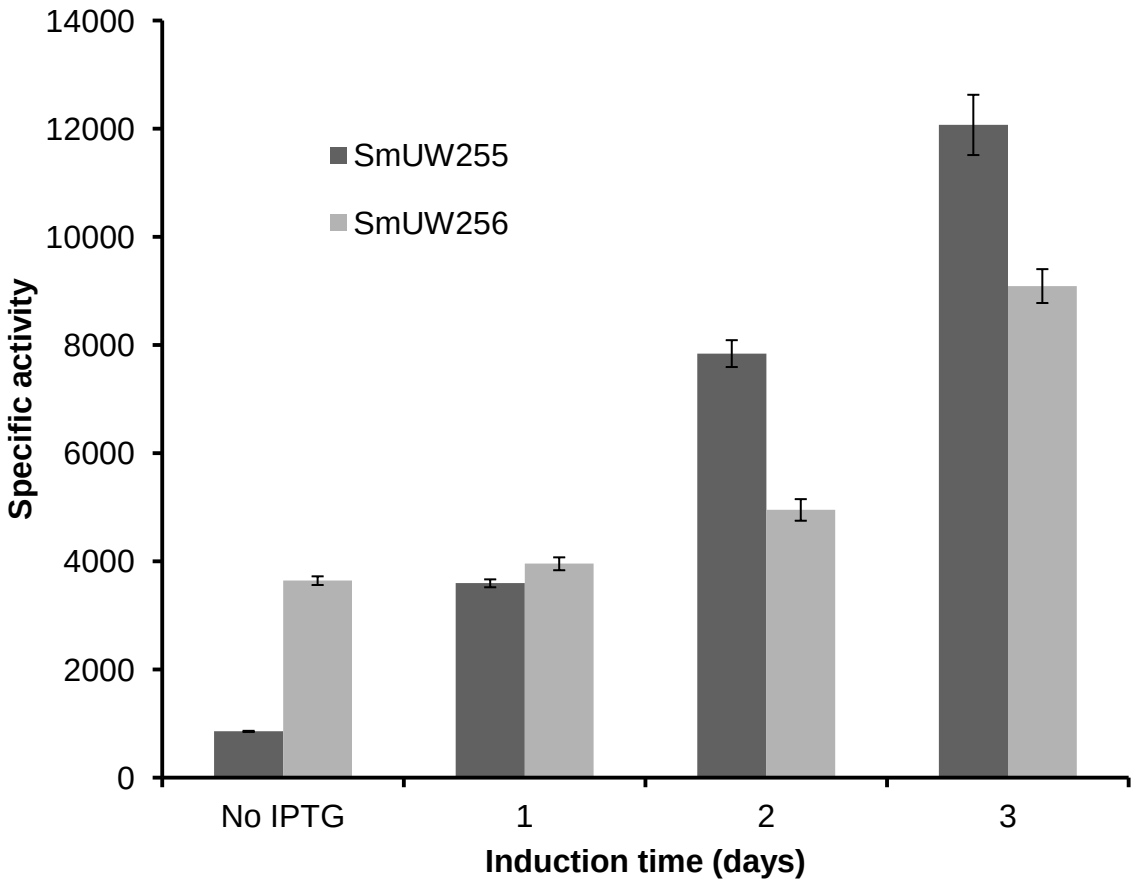


Figure 4.6 Measurement of *gusA* activity of strain SmUW256 induced at 0.4 mM IPTG at different points in time for 3 day incubation. The experiment was done in duplicate. Error bars represent the range of the mean.

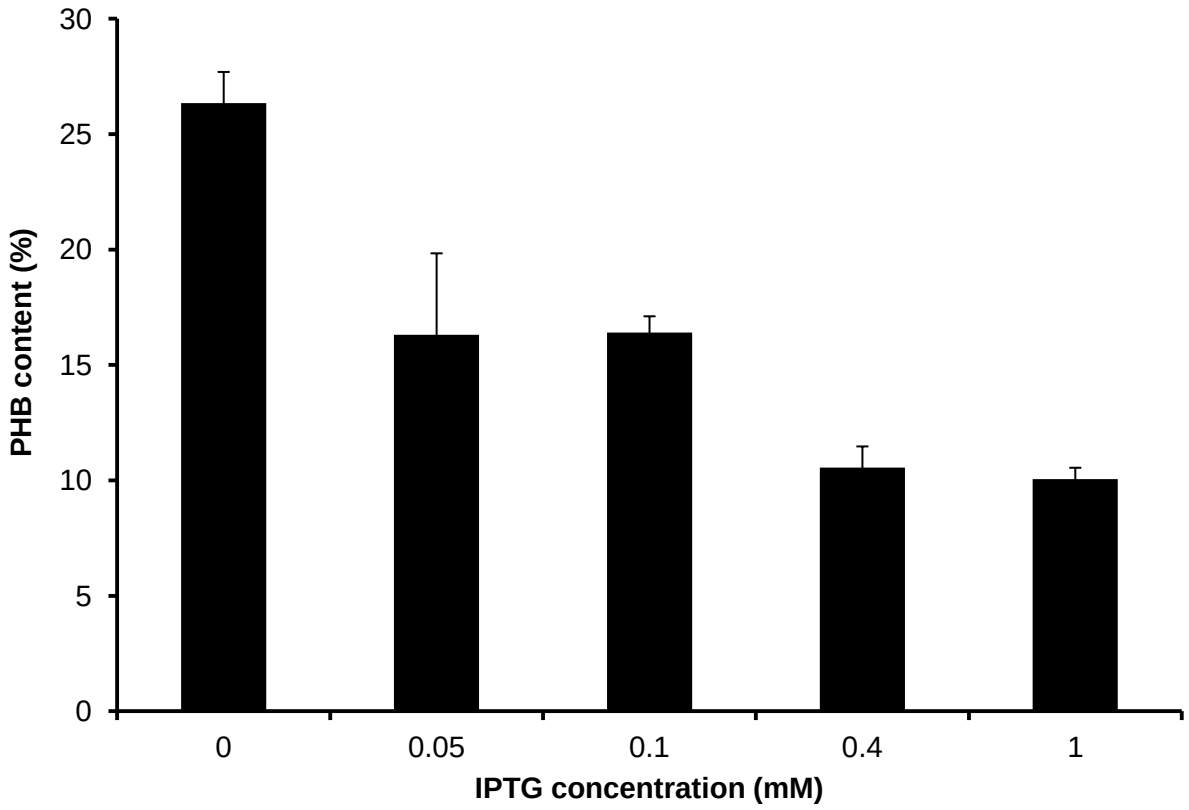


Figure 4.7 P(3HB) content produced by strain SmUW256 induced at different concentrations of IPTG. The experiment was done in duplicate. Error bars represent the range of the mean.

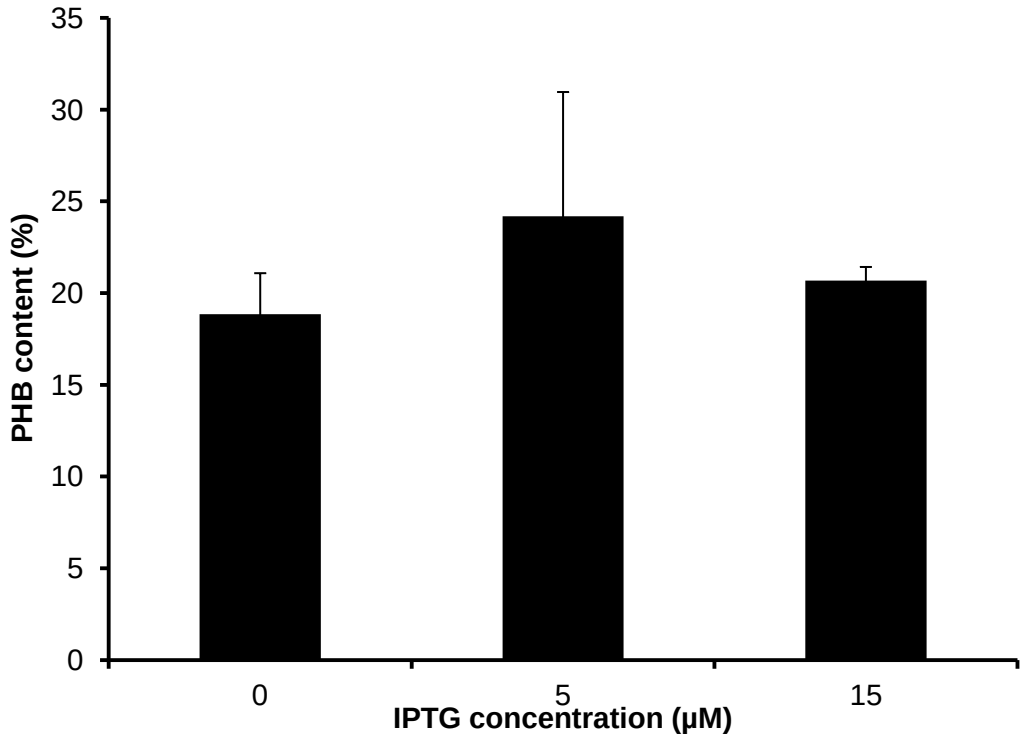


Figure 4.8 P(3HB) content produced by strain SmUW256 induced at a lower range of IPTG concentration. The experiment was done in duplicate. Error bars represent the range of the mean.

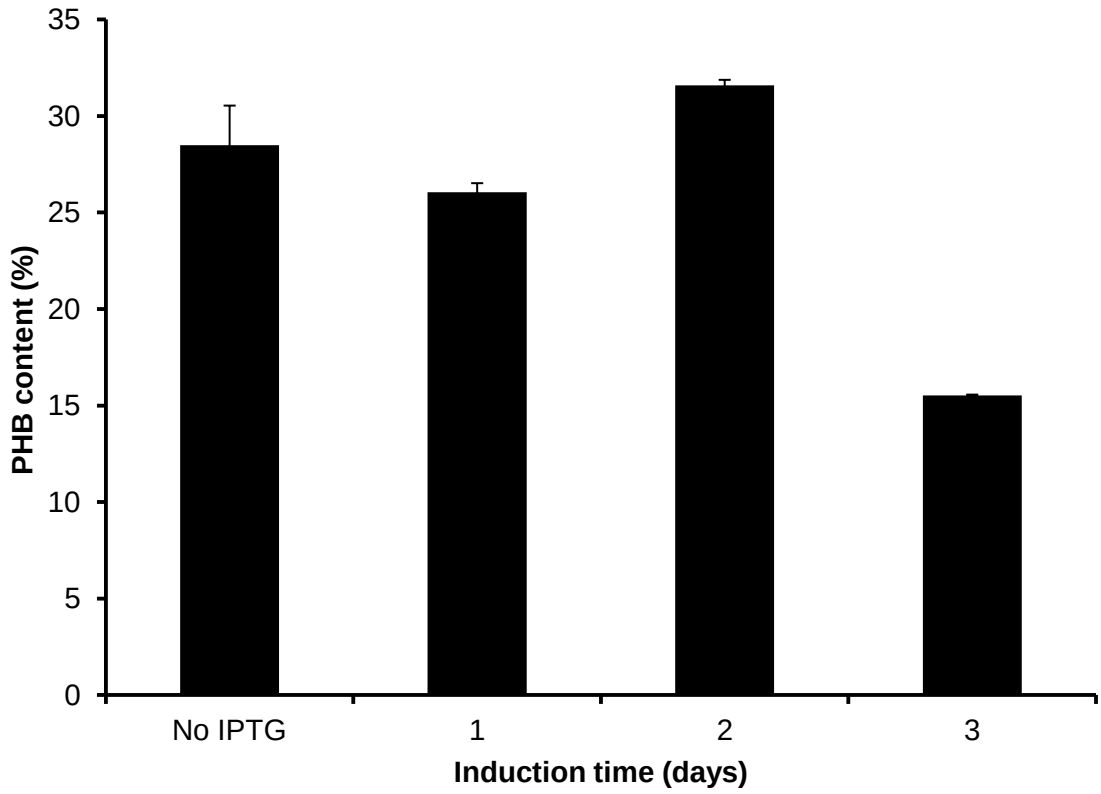


Figure 4.9 P(3HB) content produced by strain SmUW256 induced 0.4 mM IPTG at different points in time for 3 day incubation. The experiment was done in duplicate. Error bars represent the range of the mean.

Table 4.1 PLA production in *phaAB phaC* mutant (SmUW501 background) harbouring pTAM

	Lactic acid concentration					
	10 g/l	20 g/l	30g/l	10 g/l	20 g/l	30g/l
Strain	PLA content (%)			DCW (g/l)		
SmUW558 (control)	ND	-	-	0.43 ± 0.03	-	-
SmUW559	3.2 ± 1.8	0.18 ± 0.13	ND	0.35 ± 0.05	0.16 ± 0.05	ND

Strains were cultured in YEM supplemented with lactic acid at different concentrations. SmUW558: *ΔphaAB ΔphaC* (pTH1227), SmUW559: *ΔphaAB ΔphaC* (pTAM). - : Not available. ND: Not detectable

Table 4.2 Polymer production in different mutant *S. meliloti* backgrounds

Strains	Carbon source			
	Mannitol (10 g/l)		Lactic acid (10 g/l)	
	DCW (g/l)	P(3HB) (%)	DCW (g/l)	PLA (%)
SmUW550	0.39 ±0.009	nd	0.48 ±0.01	nd
SmUW551	0.6 ± 0.069	nd	0.52 ± 0.06	nd
SmUW552	0.47 ±0.032	nd	0.42 ±0.007	nd
SmUW553	0.45 ± 0.02	nd	0.62 ± 0.058	nd
SmUW554	0.34 ±0.04	nd	-	-
SmUW555	0.48 ± 0.005	7.18 ± 1.03	-	-
SmUW556	0.66 ± 0.05	14.9 ± 1	-	-
SmUW557	0.73 ± 0.04	13.9 ± 0.45	-	-

Strains were cultured in YEM supplemented with either mannitol or lactic acid. SmUW550: *ΔphaAB* (pTH1227), SmUW551: *ΔphaAB* (pTAM), SmUW552: *ΔphaAB ΔphaZ* (pTH1227), SmUW553: *ΔphaAB ΔphaZ* (pTAM), SmUW554: *ΔphaC ΔphaZ* (pTH1227), SmUW555: *ΔphaC ΔphaZ* (pTAM), SmUW556: *ΔphaZ* (pTH1227), SmUW557: *ΔphaZ* (pTAM). For full description of strains, please refer to Table 2.1.

4.3 Conclusions

In this chapter, we have demonstrated that the synthesized *pct* - *phaC* was able to complement the *phaC* mutant strain. However, the flow of LA precursor is apparently insufficient in this system, resulting in the strains-accumulating only P(3HB). It was also found that the enzyme activities did not proportionally correlate with polymer accumulation, and the strain produced P(3HB) the most efficiently at the background level. The strain that had the P(3HB) production pathway completely removed was able to produce PLA homopolymer up to 4% when growing in YEM supplemented with lactic acid.

Chapter 5 Metabolic Engineering in *Pseudomonas putida* background

5.1 Introduction

Pseudomonas putida is classified within the Gammaproteobacteria, and are Gram-negative, chemoorganotrophic aerobic rods with polar flagella. *P. putida* can naturally produce MCL PHA either from non-related carbon sources such as glucose, or related carbon sources such as fatty acids. It can accumulate PHA up to 75% DCW under carbon-limiting conditions (Le Meur et al., 2012). Also, the complete genome sequence of *P. putida* KT2440 is available, and the strain is genetically amenable, which is beneficial for manipulating metabolic pathways (Timmis, 2002). It grows fast and can be found in many terrestrial and aquatic ecosystems. For these reasons, this strain has become a very promising candidate to be a robust host for novel polymer production.

MCL PHAs are composed of monomers that contain 6-14 carbons. They are produced as intracellular storage compounds in many *Pseudomonas* strains under unbalanced growth conditions (Le Meur et al., 2012; Sudesh et al., 2000). The 3-hydroxyacyl-CoA precursors are derived from the intermediates of the fatty acid β-oxidation pathway during growth on fatty acids under nitrogen limitation. In addition, these precursors also can often be obtained through fatty acid *de novo* biosynthesis from unrelated carbon sources such as carbohydrates. Depending on the composition as well as the length of the alkyl side chain, material properties vary to a great extent from hard crystalline plastics to elastic rubbers. MCL PHAs exhibit the properties of low-melting points, high elasticity and thermoelastomers (Jiang et al., 2006).

The objective of this study was to take advantage of the existing copolymer plasmid expression system that was demonstrated in *S. meliloti*, and investigate its use in *P. putida*, a well-known MCL PHA producer. A primary goal was to combine the plasmid-based system and the native *P. putida* system to produce a novel type of polymer. The engineered PhaC synthase was shown, in *S. meliloti*, to be able to accept SCL PHA, specifically 3HB, as well as LA monomers. This enzyme is derived from the Type II PhaC synthase from *Pseudomonas* sp. MBEL 6-19 strain, and consistent with this origin it could also accept MCL PHA monomers. By replacing the *P. putida* PhaC synthase with this engineered enzyme, a novel polymer composed of 3HB, LA and different types of MCL PHA might be produced. It was demonstrated that improved properties have been achieved by blending different types of polymers to meet a wider range of practical applications (Koning, 1995; Sudesh et al., 2000). In the search for such a novel polymer, this strategy could result in the generation of new types of polymer which possesses valuable and useful properties.

5.2 Results

5.2.1 Introduction engineered synthesized genes into *P. putida* and expression under control of an inducible promoter

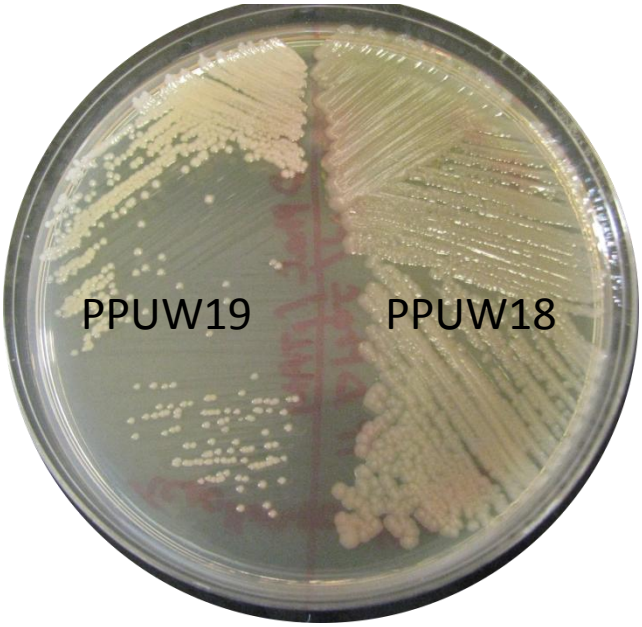
All of the strains used in this chapter are listed in Table 2.1. The pTAM vector that was constructed and expressed in *S. meliloti* as described in Chapter 4 was introduced into both the *phaC1-phaZ-phaC2* mutant and the wild-type *P. putida* KT2440 strain by triparental conjugation; the new strains were named as PPUW19 and PPUW21, respectively. The empty plasmid pTH1227 was also introduced into the mutant and wild-type strains, and the resulting strains were named as PPUW18 and PPUW20 . Since we observed an adverse effect on polymer production in *S. meliloti* under inducing conditions, these genes were expressed under *pTac* promoter in the absence of IPTG in *P. putida*.

5.2.2 Phenotypic analysis of strain constructs

The phenotype of *P. putida* harbouring the expression plasmid was taken into consideration when the strains were grown on LB supplemented with sodium octanoate. Interestingly, we found that the strains carrying the pTAM plasmid showed a distinguishable phenotype from the strains only carrying the empty plasmid pTH1227 (Figure 5.1). Both the wild type and mutant strains containing the pTAM plasmid showed a milky white colony phenotype, while the strains that carried only the empty plasmid pTH1227 had a yellowish colony color. The milky white colour is likely due to polymer accumulation enabled by pTAM. *P. putida* KT2440 is known to only accumulate PHA under unbalanced growth conditions, but to our knowledge, there is no study investigating the ability to accumulate polymer when cultured in complex media. It appears that the function of the native PhaC synthase, and not other enzymes in the *P. putida* PHA biosynthesis pathway, is affected when the strain is cultured in nutrient media with

excess carbon source because the engineered PhaC synthase was able to direct the accumulation of PHA on complex media.

We also investigated colony fluorescence on Nile Red containing plates with different growth media (Figure 5.2). On LB plates without the addition of any extra carbon source, no fluorescence was observed for any of strains. Only strains harbouring pTAM plasmid showed a strong fluorescence when grown on LB plates with an excess carbon source (eg. sodium carbonate). Meanwhile, there were no evident phenotypic differences when the strains grew on either only LB or LB with lactic acid.



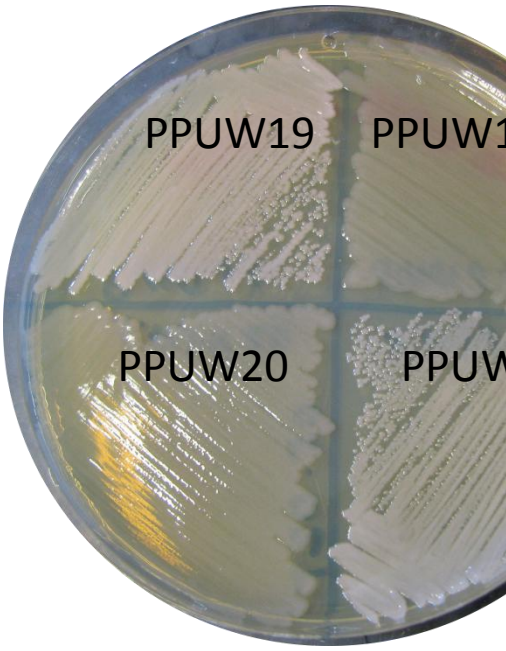
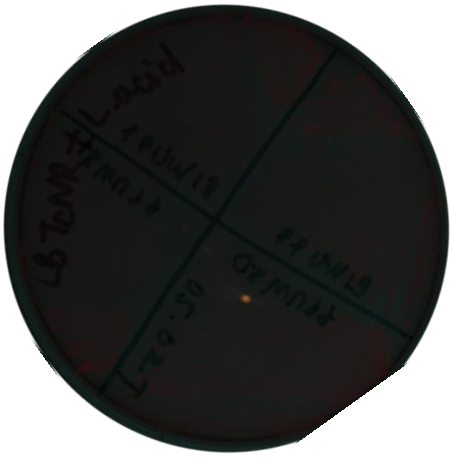


Figure 5.1 Phenotype of strains on LB supplemented with sodium octanoate. A milky white phenotype was observed for PPUW19 (*phaC* mutant harbouring pTAM) and PPUW 21 (wild-type strain harbouring pTAM). Yellowish phenotype was observed for PPUW18 (*phaC* mutant harbouring pTH1227) and PPUW20 (wild-type strain harbouring pTH1227)

LB + NR

LB + Sodium octanoate + NR



LB + Lactic acid + NR

Figure 5.2 Fluorescence observation of different *P. putida* strain backgrounds. PPUW18 (*phaC* mutant harbouring pTH1227), PPUW19 (*phaC* mutant harbouring pTAM), PPUW20 (wild-type KT2440 harbouring pTH1227), PPUW21 (wild-type KT2440 harbouring pTAM).

5.2.3 GC analysis of polymers produced in *P. putida* strains growing in minimal media supplemented with either sodium octanoate and lactic acid as a substrate

Strains were cultured in defined media 0.5E2, whose recipe can be found in the Appendix, supplemented with either sodium octanoate or lactic acid. The negative control strain PPUW18, which is the *P. putida phaC* mutant strain carrying the empty plasmid pTH1227 was not able to produce polymers. Overall, the media containing sodium carbonate as sole carbon source supported both the growth and the capability of polymer production much better than media with using lactic acid as sole carbon source (Figure 5.3). The mutant with pTAM plasmid showed the highest DCW and polymer content up to 1.28 g/l and 42%, respectively, using sodium octanoate. Remarkably, the strains carrying the pTAM plasmid were able to incorporate LA monomers to generate a novel polymer composed of LA, 3HB, 3HHx and 3HO. A copolymer of this type has, to our knowledge, never been reported. The production of P(LA-*co*-3HB-*co*-3HHX) was previously demonstrated in *E. coli* via a reverse reaction of the β-oxidation pathway (Shozui et al., 2010a). In that study, the *E. coli* strain was equipped with LA-polymerizing enzyme (LPE) encoding a mutant *phaC1* gene from *Pseudomonas* sp. 61-3, propionyl-CoA transferase (PCT) and (R)-specific enoyl-CoA hydratase (PhaJ4). However, it is difficult to understand how, in that particular study, 100% P(3HB) was produced during growth in LB supplemented with glucose since there is no link between the fatty acid synthesis pathway with PHA precursor formation in *E. coli.* In previous studies, *E. coli* was able to produce a small amount of MCL PHA from non-related carbon source only when provided with a PHA polymerase and a modified thioesterase I (Klinke et al., 1999). In addition, the polymer content of P(LA-*co*-3HB-*co*-3HHX) produced in *E.coli* was extremely low (<5% DCW).

We found that lactic acid supplied more LA precursors than sodium octanoate; therefore both the wild-type strain and the mutant strain carrying pTAM accumulated LA of 2.8 and 1.9% mol. Lactic acid is a direct substrate of the modified propionate-CoA transferase, whose function is to add the CoA moiety onto the substrate. Even though lactic acid did not support the cell growth as well as sodium octanoate, it supplied more LA precursor toward novel polymer production. This implies that the *P. putida* system did not produce lactyl-CoA efficiently from the sodium octanoate substrate.

5.2.4 GC analysis of polymers produced in *P. putida* strains growing in LB supplemented with either sodium octanoate or lactic acid as a substrate

Since we observed different phenotypic characteristics on LB agar supplemented with sodium octanoate as described above, we wanted to examine, by GC analysis, what specific types of polymers were produced. As we expected, the polymer production reflects what we observed on the agar plates (Figure 5.4). All the strains produced a relatively low amount of polymers when grown in LB supplemented with lactic acid as a sole carbon source. This is the reason that we observed very low fluorescence on the plate. The *phaC* mutant strain harbouring pTAM produced the most polymer content (3.3% DCW) and LA monomer fraction (4% mol) in lactic acid supplemented LB. This LA monomer fraction was also found to be the highest obtained among all culture conditions. The wild-type strain harbouring the empty plasmid hardly produced any polymer in nutrient media regardless of what carbon source was used. However, surprisingly, the introduction of the pTAM plasmid restored polymer production up to 25.3% DCW in LB supplemented with sodium octanoate. This could be explained by inhibition of the native PhaC in nutrient media, rendering it unable to polymerize precursors. The heterologous enzyme, nevertheless, was perhaps able to overcome such inhibition. This also implies that the

flow of precursor molecules was always continuous in both defined and nutrient media as long as there was abundant carbon source.

It has been suggested that the engineered PhaC enzyme has a broader substrate range than the native *P. putida* PhaC. The engineered PhaC synthase was originally Type II PhaC1 synthase that accepts and polymerizes MCL-3HA (C6-C14) monomers (Yang et al., 2010). This enzyme was engineered to broaden the substrate range towards SCL-3HAs (specifically, 3HB) and LA. Nonetheless, whether it still retains its ability to accept MCL-3HA has not previously been demonstrated. The fraction of LA and 3HB monomers in the copolymer increased substantially with the presence of engineered PhaC synthase, irrespective of media. This fraction was increased in mutant PhaC strain to a greater degree than the strain that still contained the wild type *phaC* genes, probably because either there is precursor competition between the polymerase enzymes in the wild-type strain or the overall polymerase enzyme activity towards MCL PHAs is higher in the wild-type resulting in the increase of MCL fraction over LA and 3HB fraction. In conclusion, we demonstrated that enhanced activity has been achieved, in which not only SCL-3HA and LA monomers but also MCL-3HA monomers have been accepted by this enzyme.

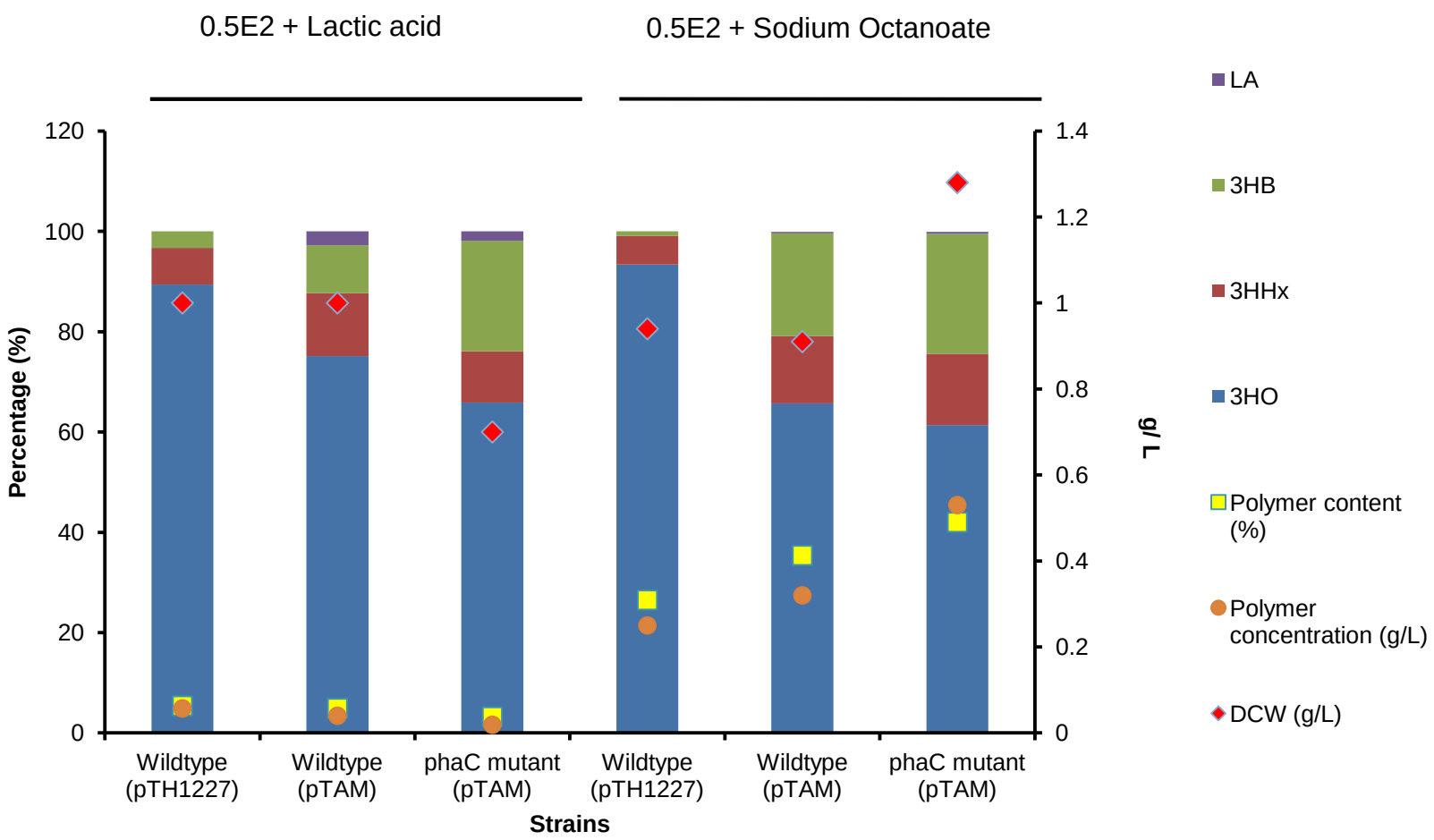


Figure 5.3 Polymer production in different *P. putida* backgrounds growing in 0.5E2 media supplemented with either lactic acid or sodium octanoate. Left axis represents the polymer content (yellow squares) and monomer composition (bars). Right axis represents polymer concentration (g/l) and DCW (g/l).

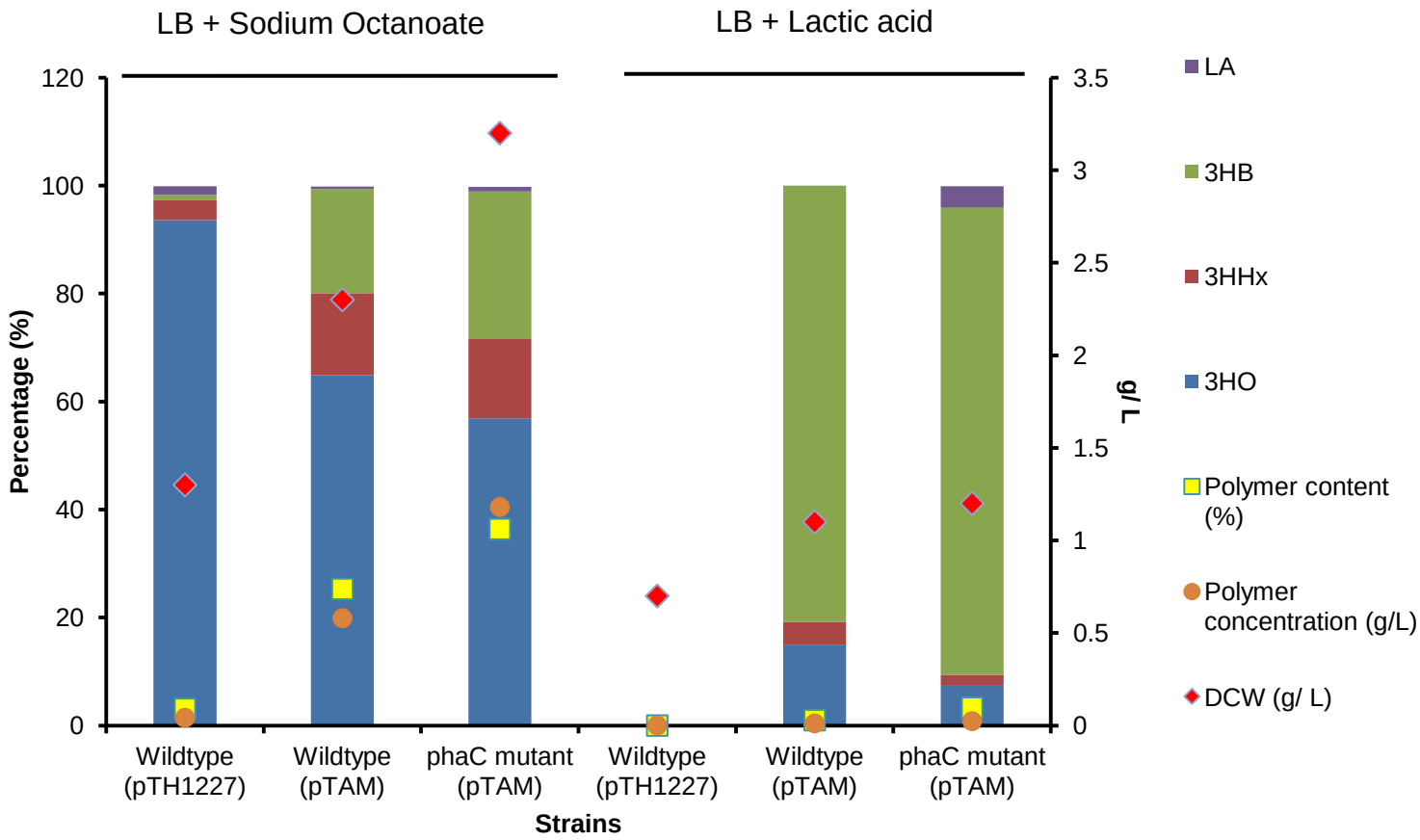


Figure 5.4 Polymer production in different *P. putida* backgrounds growing in LB media supplemented with either lactic acid or sodium octanoate. Left axis represents the polymer content (yellow squares) and monomer composition (bars). Right axis represents polymer concentration (g/l) and DCW (g/l).

5.3 Conclusions

Here we have demonstrated that the plasmid expression system worked well in a different platform other than *S. meliloti*. A new type of polymer P(3HB-*co*-LA-*co*-3HHx-*co*-3HO) has been produced by expressing modified genes in *P. putida*. The *phaC* mutant strain produced this type of polymer the most efficiently with the polymer content of 36.4% DCW when cultured in 0.5E2 with the addition of sodium octanoate, while the greatest LA fraction (4 mol%) was achieved in the same strain cultured in LB with the addition of lactic acid. Polymer production, specifically native PhaC polymerase activity, is dependent on the type of media used. The nutrient media LB supplemented with sodium octanoate appeared to repress the enzyme activity of native PhaC, but not engineered PhaC. The LA and 3HB monomer composition was higher when engineered PhaC was expressed alone than when engineered PhaC was co-expressed with native PhaC.

Chapter 6 Promoter Engineering

6.1 Introduction

Promoter engineering has been considered as one of the critical strategies in the metabolic engineering field. A number of studies have been carried out to predict and characterize the promoter strength using *in silico* and/or wet-lab experiments (Davis et al., 2011; MacLellan et al., 2006; Murphy et al., 2007; Rhodius and Mutalik, 2010). Strong promoters are normally desired to maximize the end products, which could be proteins, amino acids, polymers or biofuel, and reduce toxicity during the growth phase. However, promoter strength and optimal yield of end products are not always correlated proportionally; therefore, promoters that are fine-tunable and tightly controlled are more beneficial and versatile. The inducible promoter Ptac has been employed and presented in previous chapters. Here we present another approach to obtain a broad range of constitutive promoter strengths with no inducer required

6.1.1 The structure of polymerase and bacterial transcription

The process of transcription in bacteria is comprised of three steps: initiation, binding and elongation. The structure of bacterial RNA polymerase which is a multi-subunit complex is normally composed of core components (ββ'αω) and one of several σ subunit types. The σ subunit is responsible for initiating transcription via searching and binding to two hexameric DNA sequences at the -10 and -35 positions. These positions contain conserved sequences known as motifs that are found in numerous promoters; for example, consensus sequence in *E. coli* promoters are –35 “TTGACA” motif and a –10 “TATAAT” motif (Blazeck and Alper, 2013). These motifs are separated by a non-conserved sequence of roughly 17 base pairs. Besides promoter elements located at -10 and -35 position, there is the participation of the third

element (UP element) which consists of AT rich sequence residing upstream of -35 region. This third promoter element interacts with the C-terminal domain of the α subunit and can act as promoter facilitator to enhance the promoter activity (Meng et al., 2001). Upon binding, the double-stranded DNA sequence from approximate positions -10 to +2 is melted and separated into single strands to form the open complex and initiate the transcription. Before the polymerase is able to successfully proceed to elongation, it sometimes goes through a repetitive process, known as abortive transcription, in which the polymerase produces a short RNA transcript (<10 nt) and returns to the start site until it is able to escape from the σ subunit and move forward. To learn more about the relationship between σ subunit structure and abortive transcription, σ^{70} was mutated by changing a single amino acid in the conserved protein region 3 (Hernandez et al., 1996). It was found that a pattern of abortive RNAs was qualitatively different between the mutants and the wild-type σ. A similar level of short abortive RNAs was detected for mutants and wild-type enzyme, but longer abortive RNAs were produced at greater or lesser amounts by the mutant enzymes depending on promoter specificity, RNA length or promoter distance. Normally the maximum length of abortive transcripts is 10 nt; however there could be some exceptions which produced longer abortive transcripts up to 16 nt. Surprisingly, promoter clearance seemed slightly affected by the σ mutants suggesting that the process of abortive RNA production might not be tightly associated with that of promoter clearance.

6.1.2 Application of promoter engineering in gene expression

To study gene expression or the effect of a specific promoter on gene expression, a reporter gene located downstream of the promoter is normally used. There are various reporter genes which have been employed to detect gene expression such as *gfp* or *gusA* genes (Alper et al., 2006; Leveau and Lindow, 2001; MacLellan et al., 2006; Myronovskyi et al., 2011; Rhodius

and Mutalik, 2010). A library of engineered promoters of different strengths has been constructed through either mutagenesis of constitutive promoters or chemical synthesis of promoter variants (Alper et al., 2006; Jensen and Hammer, 1998; Jørgensen et al., 2004). By changing the length and the sequence of the spacer between the -35 and -10 regions, a broad range of promoters which differ in strength has been studied in *Lactococcus lactis.* Randomizing the spacers resulted in a remarkable change in activities up to 400-fold (Jensen and Hammer, 1998). It was also emphasized that the context in which the consensus sequences are embedded significantly influences the promoter strength. In a subsequent study, this strategy has been employed to obtain the expression of the *pyrG* gene encoding CTP synthase at different levels and its effect on the growth rate and nucleotide pool size (Jørgensen et al., 2004). Another library of nearly 200 promoters was also obtained using error-prone PCR to examine gene expression in *E. coli* via using *gfp* as a reporter gene (Alper et al., 2006). These promoter strengths were also assessed by testing the effect of the expression of the *ppc* gene encoding phosphoenol pyruvate (PEP) carboxylase on growth yield and encoding deoxy-xylulose-P synthase on lycopene production. It was suggested that the optimization of gene expression could depend on the genetic background of the strain.

The objective of this strategy is to replace a single inducible promoter with a broad range of native promoters of different strengths. Although inducible promoters allow for tuning gene expression at various levels of inducers, using this system might limit practical applications at an industrial scale due to expensive inducer costs, heterogeneities in cellular response and difficulty in optimizing inducer concentration and inducing time as well as maintaining a steady expression level of the enzyme (Alper et al., 2006; Jensen and Hammer, 1998). Therefore, we would like to

employ a wide range of constitutive promoters in strength to examine their effect on polymer production.

6.2 Results and discussion

6.2.1 Construction of plasmids with promoters of different strengths

A range of promoters of different strengths (6 out of 12 native promoters) based on a previous study (MacLellan et al., 2006) was chosen for further investigation. These promoter sequences (Table 2) were synthesized in the form of extended primers that were annealed to create double-strand DNAs. The backbone plasmid, pTH1227a, is a derivative of pTH1227 that had the XbaI site removed by being digested, blunted and self-ligated as shown in Figure 6.1. Promoter P_0 was designed to contain XbaI site next to HindIII site, and cloned into pTH1227a at the HindIII and XhoI sites, creating pTH1227b. Next, the synthesized fragment which contains two engineered genes were inserted into the pTH1227b at the XhoI and PstI sites. The rest of the promoter set containing BglII for post-cloning confirmation was cloned into XbaI and XhoI, replacing promoter P_0. As a result, we have constructed 6 different versions of the pTAM plasmid carrying different promoters of varying strengths.

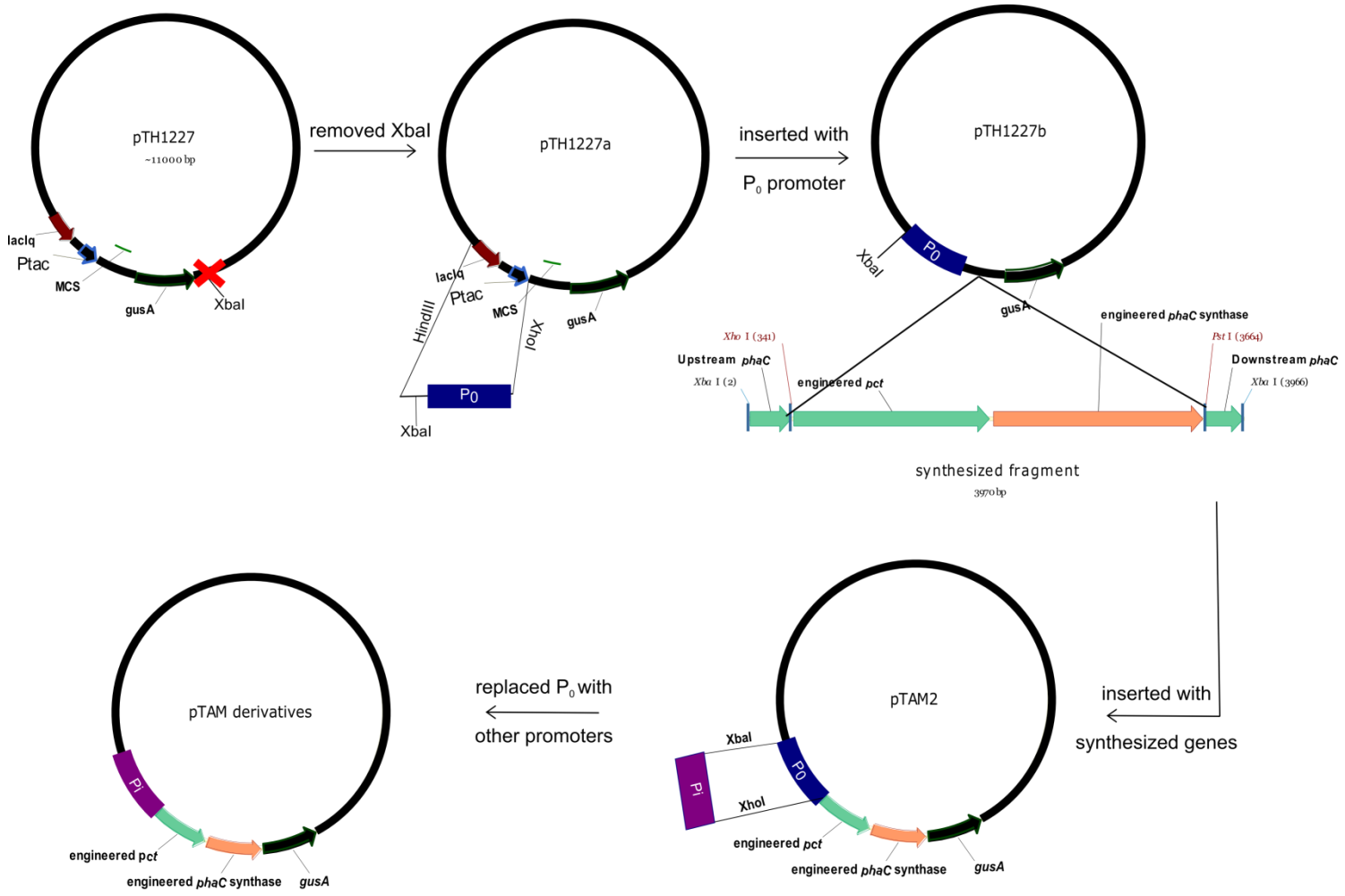


Figure 6.1 Construction of plasmids with promoters of different strengths. In the last step, P_0 was replaced with P_i which is one of five promoters (P1, P2, P3, P4 and P5).

6.2.2 GusA activities obtained by expressing *gusA* gene under a set of constitutive promoters of different strengths in the presence of synthesized genes

GusA activity assays were performed to examine the level of gene expression according to each specific promoter that was used. As shown in Figure 6.2, a wide range of GusA activity was obtained indicating that different levels of gene expression were successfully achieved. Also, in some cases the level of gene expression was even lower than the background level of gene expression obtained by using the inducible promoter. However, the transcriptional levels of some promoters in our study were completely contradictory to previously reported values. For example, we found that the pTAM7 plasmid carrying the *ropB1* promoter exhibited the highest levels of expression, while MacLellan et al (MacLellan et al., 2006) had previously demonstrated that this same promoter had the lowest level of expression among the same set of promoters. Another promoter that strongly disagreed with MacLellan et al in terms of gene expression level was the *Smc1378* gene promoter in the pTAM2 plasmid. In the previous study, expression was the highest of all of the promoters, but we found that the expression was relatively low compared to other promoters in the same set. This finding raised concerns about potential context effects on gene expression. It could be due to plasmid backbone, inserted gene sequence, the reporter gene employed or the cultivation condition. In a previous study, the relative promoter activity has been proved to remain unchanged across different cultivation conditions (Keren et al., 2013). The activities of roughly 900 *S. cerevisiae* and 1800 *E. coli* promoters are taken into consideration, and their gene expressions changed among different cultivation conditions by a constant factor. In other words, they mostly behaved alike and maintained their relative activities levels across different cultivation conditions. Therefore, the cultivation condition could be ruled out as a possible cause. Among the remaining potential causes, we postulated that inserted gene

sequence most probably caused the irrelative change in gene expression of some promoters. Therefore, we removed this inserted sequence from the plasmid construct and compared the GusA activity of the construct with and without these inserted genes.

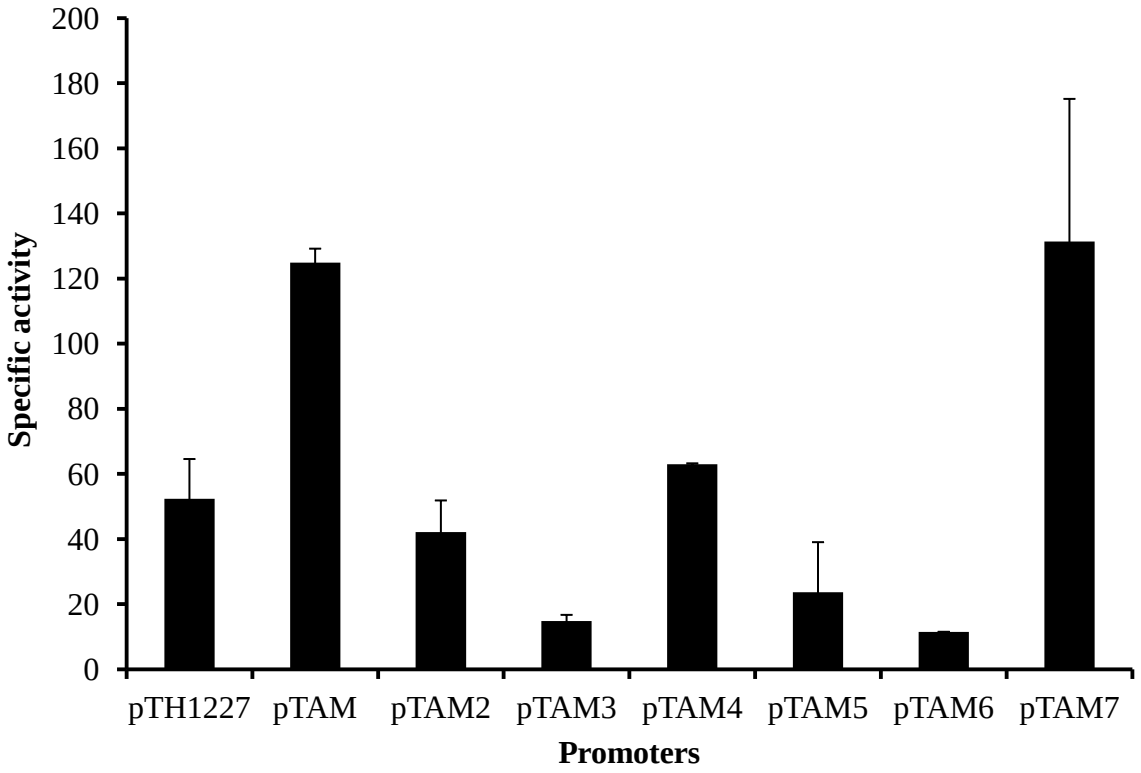


Figure 6.2 GusA specific activity of *S.meliloti phaC* mutant SmUW499 harbouring either the parental plasmid (pTH1227) or different pTAM derivatives (pTAM, pTAM2 -> pTAM7). pTH1227: empty plasmid, inducible pTac promoter; pTAM: inducible pTac promoter + insert genes; pTAM2: *smc1378* gene promoter + insert genes; pTAM3: *rpmJ* gene promoter + insert genes; pTAM4: *rpmE* gene promoter + insert genes; pTAM5: *secE* gene promoter + insert genes; pTAM6: *rpoD* gene promoter + insert genes; pTAM7: *ropB1* gene promoter + insert genes

6.2.3 GusA activities obtained by expressing *gusA* gene under a set of constitutive promoters of different strengths in the absence of synthesized genes

To further investigate the effect of the synthesized genes on the promoter strength, we chose three plasmids, pTAM2, pTAM6 and pTAM7, which carried the *smc1378, rpoD* and *ropB1* promoters, respectively. Since the expression from the *smc1378* and *ropB1* promoters was in strong disagreement with the previous study of MacLellan et al. on expression of these reporter genes while *rpoD* gene maintained the weakest activity in both our study and the previous study, we thought that it would be interesting to examine the expression of the reporter gene under these promoters in the absence of the intervening synthesized genes. Therefore, the fragment containing the synthesized genes was removed by XbaI and PstI digestion followed by self-ligation, named pTAM2no*pctPhaC*, pTAM6no*pctPhaC* and pTAM7no*pctPhaC*. Surprisingly, removal of synthesized genes recovered the original promoter activities which were reported in by MacLellan et al. for strains which have genes expressed under the *smc1378* and *ropB1* promoters (Figure 6.3). The *rpoD* promoter still maintained the low activity which was observed with and without the inserted genes. This suggested that the downstream sequence probably influenced the transcription process. Other work has shown that the downstream region can have a strong influence on the efficiency of the escape process of RNA polymerase because release of the σ subunit only occurs after the polymerase has transcribed 8-11 nucleotides (Chan and Gross, 2001; Hernandez et al., 1996). This region was earlier reported by Bujard and co-workers (Kammerer et al., 1986). They found that the down-stream sequence could change the promoter strength *in vivo* more than 10-fold. The reason is that RNA polymerase covers a larger region than 35 bp (up to 70 bp); hence, its activity also depends on the flanking regions which could be up-stream or down-stream regions. It was known that this region was involved in

promoter escape; however, the mechanism of this process was not understood. By further study of the anti initial transcription sequence (ITS) which was discovered earlier to have an effect on promoter strength, it was found that the function of the anti ITS did not depend on either the stability of RNA:DNA bond or the interaction with core RNA polymerase. It appeared that the function of anti ITS was related to the σ subunit. As a result, the interactions between them could influence the promoter escape. In other studies, it was also observed that characterized promoters often showed variable activities depending on the genetic locus or gene transcribed (Alper et al., 2006; Hammer et al., 2006; Jensen and Hammer, 1998; Kelly et al., 2009; Martin et al., 2009). A library of variable-strength, constitutive promoters was designed and constructed in bacteria (Davis et al., 2011). The length of these promoters is 160 bp including extended sequences at both the 5'- and 3'-ends, so-called insulation sequences. Their promoter strengths were shown to maintain constant relative levels and be independent of genetic contexts. From the above evidence, we reason to suspect that the initial sequence of roughly 20 bp in the synthesized gene fragment may have had some effect on promoter strengths. Since the promoter activities were indirectly assessed through the expression of *gusA* reporter gene, there is no clear evidence to prove that these observations happened at the transcriptional level, not translational level. Therefore, we continued to investigate the abundance of RNA transcripts of *pct* and *gusA* genes in the pTAM2 and pTAM7 plasmids to verify these observations occurring at the transcriptional level.

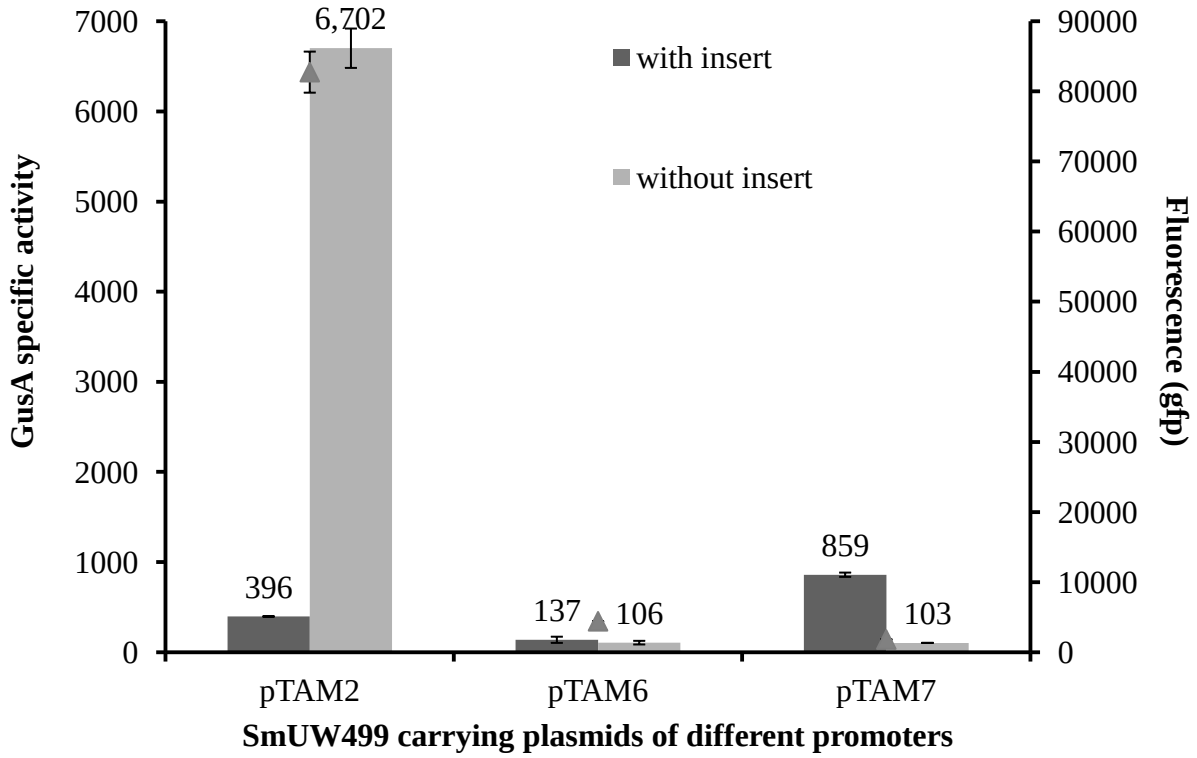


Figure 6.3 Comparison of activity of *gusA* gene (left axis) and *gfp* gene (right axis). Dark bars: *S. meliloti* strains SmUW499 carrying one of three plasmids pTAM2, pTAM6 and pTAM7 which are composed of *smc1378, rpoD, ropB1* gene promoters, respectively, and synthesized genes were measured the activity of *gusA* gene. Light bars: *S. meliloti* strains SmUW499 carrying one of three derivative plasmids pTAM2, pTAM6 and pTAM7 which are composed only *smc1378, rpoD, ropB1* gene promoters, respectively, were measured the activity of *gusA* gene. Triangle dots: the activity of *gfp* gene which were expressed under the corresponding promoter have been reported in a previous study (MacLellan et al., 2006). Error bars represent the range of the mean.

6.2.4 Analysis of transcript abundance of *gusA* and *pct* genes using dot blot

From the above results, the presence of synthesized genes that changed genetic context has shown the effect on the activity level of some promoters which were indirectly assessed by measuring *gusA* activity of the reporter gene. As shown in Figure 6.4 and Figure 6.5, the abundance of *gusA* transcripts corresponded with the GusA activity, suggesting that the presence of synthesized genes caused the decrease and increase of *gusA* transcripts for pTAM2 and pTAM7 plasmids, respectively. The abundance of *pct* transcripts was also investigated and compared to that of *gusA* transcripts on the plasmids carrying the synthesized genes (see in Appendix D). As our expectation, the abundance of *pct* transcripts is similar to that of *gusA* transcripts since they are transcriptionally fused. These results support the hypothesis that the downstream sequence influenced on the transcription process which led to the change in the abundance of transcripts and enzyme activity as a consequence. However, which sequence of the downstream region and how far is it from TSS are still not identified. As mentioned earlier, the first 20 amino acids following the TSS might play an important role in either facilitating or impeding the transcriptional process. Therefore, it would be interesting to further investigate these sequences in the future.

There have been a number of studies focusing on the effect of the -10 and -35 regions on promoter strength. However, only the upstream region before the transcription start site (TSS) has been seriously taken into consideration. In our study, it has been shown experimentally that this adverse effect exists. In the presence of engineered genes, relative promoter strength is different from what was reported. Removal of the insert fragment restored their relative strengths

consistently across different reporter genes. We also demonstrated that the change occurred at the transcriptional level of both insert fragment and reporter gene.

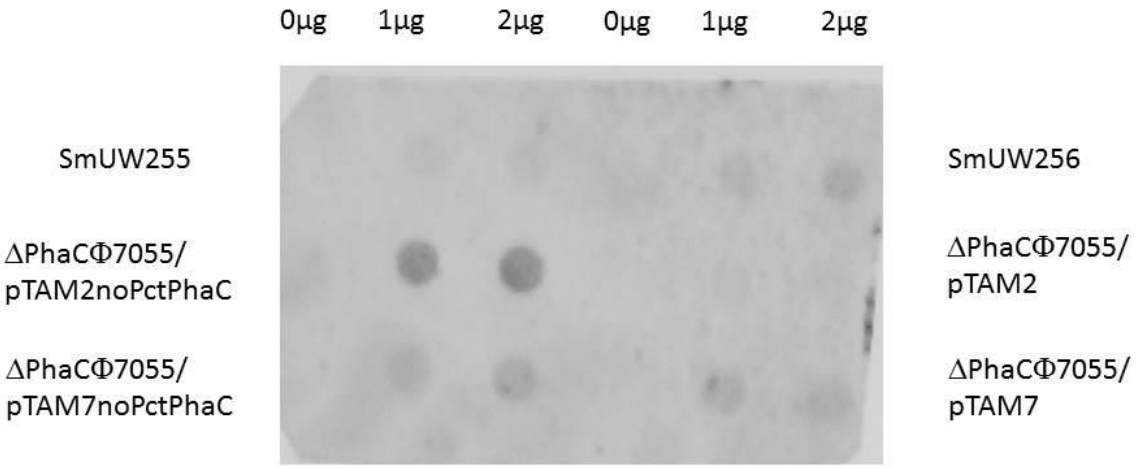


Figure 6.4 Dot blot image of *S. meliloti* SmUW499 carrying plasmids of different promoters using *gusA* probe. Three left columns represent RNA samples of different amounts (0, 1, 2µg) of SmUW255 (SmUW499 (pTH1227)), SmUW564 (SmUW499 (pTAM2no*pctphaC*)), SmUW567 (SmUW499 (pTAM7no*pctphaC*)). The other right three columns represent RNA samples of different amounts (0, 1, 2µg) of SmUW256 (SmUW499 (pTAM)), SmUW558 (SmUW499 (pTAM2)), SmUW563 (SmUW499 (pTAM7)).

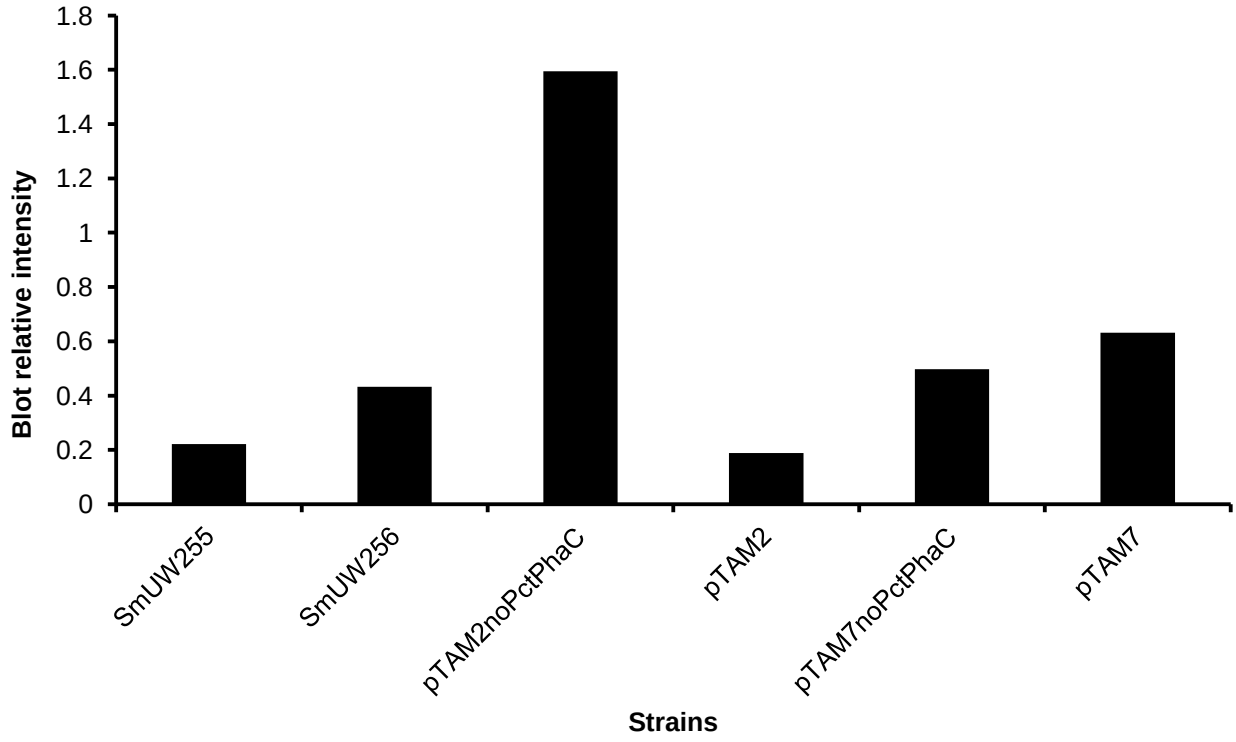


Figure 6.5 Graph represents the relative intensity on dot blot results as shown in Figure 6.3

6.2.5 Polymer production in strains that have synthesized genes expressed under these constitutive promoters

To evaluate the different expression profiles on polymer production, these strains were cultivated in define media supplemented with mannitol as a substrate and grown under polymer accumulating conditions. As expected, strains carrying pTAM6 and pTAM7, which had the weakest and strongest promoter strengths produced the least and most amount of PHB, respectively (Figure 6.6). The strains carrying pTAM7 which showed promoter strength similar to that of the strain carrying the *tac* promoter under induction conditions also accumulated a similar amount of PHB. Even though other strains showed a range of promoter strengths, they all produced rather low amount of polymer. Unfortunately, none of them was able to produce copolymer P(3HB-*co*-LA). Nevertheless, we showed that a range of constitutive promoters could be used to regulate the accumulation of polymers.

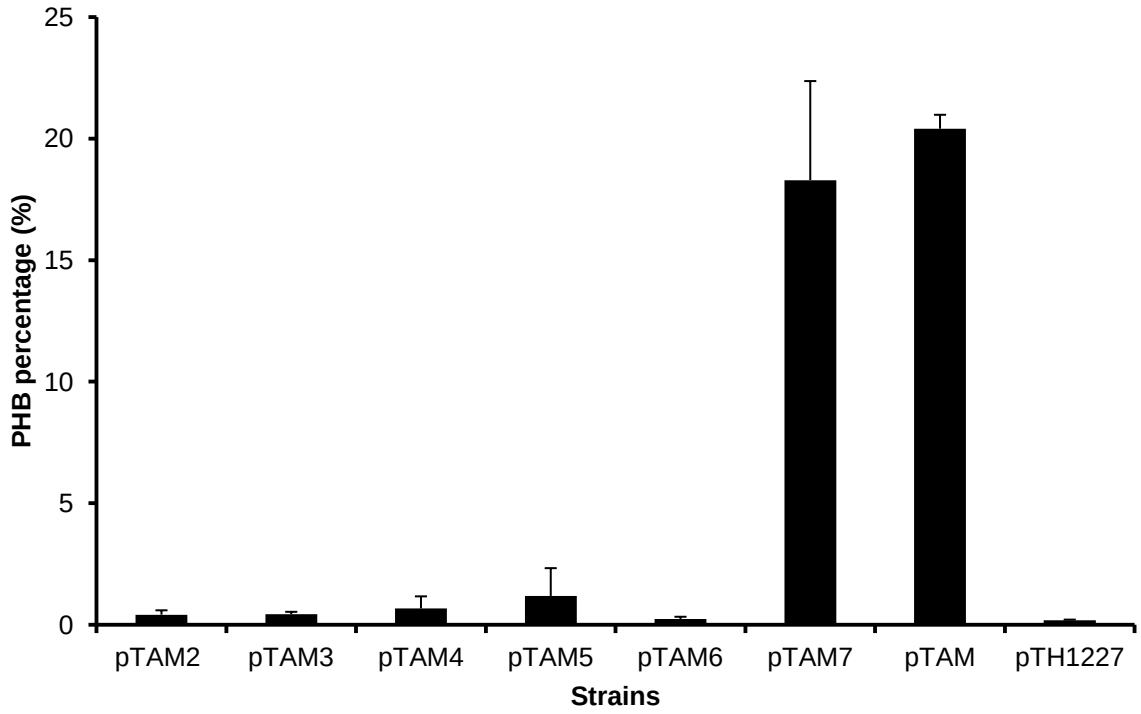


Figure 6.6 P(3HB) production in *S.meliloti* phaC mutant strains SmUW499 that have synthesized genes are expressed under different native promotersP(3HB) production in *S.meliloti phaC* mutant strains SmUW499 that have synthesized genes are expressed under different native promoters (pTAM2 -> pTAM7) and inducible promoter (pTAM); the negative control *S.meliloti* SmUW499 strains carrying the empty plasmid pTH1227. Error bars represent the range of the mean.

6.3 Conclusions

In this chapter, we focused on studying the effect of different native promoter strengths on polymer production. As shown in the previous chapter, polymer production using an inducible promoter was unpredictable and out of beyond our expectation. In addition, the future prospective does not support inducer usage in large-scale fermentation. Therefore, we selected a range of promoters of different strength that were well-characterized in the literature. However, our study did not agree with previous study, in which the relative promoter strengths have varied not correspondingly. We found that the inserted gene located close to promoters had a significant effect on promoter strengths. We postulated that this effect response may be due to effects on the occurred during the releasing process of RNA polymerase from the σ subunit.

Chapter 7 Conclusions

7.1 Research summary

We have partially codon-optimized two engineered genes, the *pct* gene encoding propionate CoA-transferase and the *phaC* gene encoding PhaC synthase and introduced them into *S. meliloti*. They were either integrated into the *S. meliloti* chromosome and expressed under native promoter or introduced into the broad-host range expression vector pTH1227 and expressed under the inducible promoter pTac. The *pct* gene which was originally from *Clostridium propionicum* had an increase of GC content from 40.38% to 53.65% after codon-optimization. Meanwhile, the *phaC* gene which was originally from *P. putida* did not show any drastic change of GC content (62.14% compared to initial 58.86%) after codon-optimization since *P. putida* has GC content similar to *S. meliloti*. In contrast, the overall number of codons drastically decreased across both genes (for the *pct* gene, the number of codons decreased from 52 to 37; for the *phaC* gene, the number of codon decreased from 56 to 39).

For the chromosome engineered system, the strain exhibited the mucoid, milky white phenotype, similar to the phenotype of the wild-type strain. The *phaC* mutant strain which lacks the ability to polymerize 3HB-CoAs showed a distinctly non-mucoid phenotype. To further analyze the types of polymers produced, the strain was cultured in defined media supplemented with mannitol and subsequently subjected to put through GC-FID analysis process. The copolymer P(3HB-*co*-LA) was found to be produced up to about 15% in the strain. To confirm the chemical structure of this compound, we also purified the polymer and analyzed it using both GC/MS and NMR. Both experiments gave results consistent with the whole-cell analysis results

using GC-FID. The advantage of this system is that genes are permanently maintained in the cell without the risk of losing genes and there is no need to add antibiotics which are an added cost.

For the plasmid-based engineering system, two codon-optimized genes were placed on a broad-host range vector pTH1227 and expressed under an inducible promoter pTac-*lacI*^{q}. In this system, we analyzed the polymer produced in both inducing and non-inducing conditions. We expected that this system would provide us a tool to finely tune the expression of genes and consequently polymer production. The addition of IPTG inducer did increase the expression of the synthesized genes which was indirectly assessed by measuring the activity of the *gusA* reporter gene. The pTac-*lacI*^{q} has been reported in *E. coli* system, to exhibit no detectable or very little leakiness. However, this promoter in *S. meliloti* appears not to be repressed as efficiently as in *E. coli*. We also examined the Nile red fluorescence . Nile red is known as a non-specific polymer indicator because the strains producing polymer exhibit fluorescence upon binding of polymers to Nile Red. We found that at the background level in the absence of inducer, the strains still exhibited fluorescence. More interestingly, the greater the induction with IPTG, the less polymer was found to be produced. However this has been reported in previous studies in which the enzyme activity did not proportionally correspond to the amount of the target products as previously discussed in Chapter 5. It would be necessary to study in-depth about the relationship between enzyme kinetics and the mechanism of polymer production. Unfortunate, there is no P(3HB-*co*-LA) accumulated using this system in the *phaC* mutant *S.meliloti* strain. We also tested this system in different *S. meliloti* mutant backgrounds (*ΔphaZ*, *ΔphaAB*, *ΔphaAB+Z*, *ΔphaC+Z*); they too, however, were also unable to produce any P(3HB-*co*-LA). Once we blocked the pathway that produces 3HB-CoAs which are the direct precursors of P(3HB), about 4% PLA was produced in the *phaABC* mutant harbouring pTAM when cultured

in defined media supplemented with lactic acid. This suggests that P(3HB-*co*-LA) copolymer was not produced efficiently in this system because the flow of LA-CoAs is too low to efficiently incorporate into growing polymers. Also, the presence of native PhaC synthase appears to impede the activity of engineered PhaC synthase resulting in no PLA accumulation; this was found in strains that had the 3HB-CoAs pathway blocked but native *phaC* still active. Since one of the advantages of this system is the flexibility, we continued exploring the outcome by applying this system in a *P. putida* background. The pTAM plasmid was introduced into both wild-type and *phaC* mutant strains, while the empty plasmid pTH1227 was also introduced into the same strains as controls. We found that only strains harbouring pTAM showed a difference phenotype and exhibited the fluorescence on LB supplemented with sodium octanoate and Nile Red. Further analysis using GC indicated that these strains were able to produce the novel polymer P(3HB-*co*-LA-*co*-3HHx-*co*-3HO) in defined media supplemented with sodium octanoate. In other words, the plasmid-based system also worked well in *P. putida;* and the engineered *phaC* gene was able to accept not only LA-CoAs and 3HB-CoAs but MCL 3HA-CoAs such as 3HHx-CoAs, 3HO-CoAs as well. We also investigated the polymer production in these strains cultured in rich-nutrient media such as LB supplemented with either lactic acid or sodium octanoate. The wild-type *P. putida* KT2440 with the empty plasmid barely accumulated polymer in this rich-nutrient media while the strains with the pTAM plasmid (either mutant or wild-type background) were able to accumulate polymer. These results suggested that the native *phaC*, not other genes in the pathway, was subject to the regulation induced by the particular culture medium that was used.

Since the inducible promoter was leaky and not economically cost-effective for an industrial scale, we have put efforts into substituting the inducible promoter with native

promoters of different strengths. These native promoter strengths have been examined in *S. meliloti* in a previous study by indirect assessment of the fluorescence of the *gfp* reporter gene. However, the promoter strengths assessed by measuring GusA activity in our study did not correspond to the results of the previous study. The strongest one that was found in the previous study has drastically dropped down the activity while the weakest one became the strongest one in our study. The downstream sequence after the TSS presumably gave rise to the observed difference in promoter activity since the removal of the insert genes brought back to the initial activity of the promoters. So we postulated that this downstream sequence, specifically the sequence of insert genes, played an important role in altering the speed of transcriptional process, technically the releasing of RNA polymerase from σ unit. In order to prove that this actually occurred at the transcriptional level, we carried out a dot blot assay and looked at the abundance of *gusA* and *pct* transcripts. As we expected, the results were consistent with the enzyme activities given by expressing the reporter gene. Therefore, the promoter strengths might be different depending on a specific context that they are put into. In addition, although promoter activities are characterized mostly based on -10 and -35 regions, we think that these regions are inadequate to define promoter strengths. We suggest that a broader range of regions including roughly 20 bp before and after this region should be also taken into account.

7.2 The importance of this study and future perspective

Waste treatment and pollution are serious environment-related issues which have adverse effect on human health. Petroleum-derived plastics are known to be extremely persistent in the environment. Therefore, there is an urgent need to find out alternative materials that can substitute these traditional plastics. Biodegradable polymers have emerged as a potential candidate because of their easy biodegradability, biocompatibility as well as their wide range of

potential applications. PHAs are a good example of biodegradable polymers that have been studied extensively and intensively for the past decades because they are naturally produced and degraded by bacteria. However, they either do not meet material requirements or cost too much for mass production. Therefore, much effort has been put into making PHAs overcome these limitations.

P(3HB) was one of the first commercialized products developed to the pilot plant stage by Imperial Chemical Industries (ICI, England) in the 1980s. However, this business was not sustainable since the production cost is high and the properties of this polymer are not superior to those of polypropylene. Based on a great deal of intensive research, the blend of 3HB with 3HV has been proved to exhibit improved properties compared to those of P(3HB) only. Therefore, in 1996, this type of polymer, which had a trade name Biopol, was invested by Monsanto who acquired the patents for making the polymer from ICI/Zeneca. However, in 2001, this company sold its rights of Biopol to Metabolix, an American company, which was planning to broaden its range of PHA products. As of 2004, all of the Monsanto's fermenters which were used for P(3HB) production were closed down. Meanwhile, Metabolix, an innovation-driven bioscience and engineering company founded in 1992, is still continuing to develop and commercialize PHA biopolymers to the market via fermentation using renewable carbon based feedstocks. According to the statement on Metabolix company, the growing demand for conventional plastics is 3-5% annually which is approximately 40 million metric tons. This raised more concerns about the scale-up accumulation of plastic waste in the environment. It was estimated that approximately 20% is the annual growth rate of increasing demand for biobased and biodegradable polymer. In addition to Metabolix, other top companies involving in the PHA business are Meredian Inc. (U.S.), Biomer (Germany), Tianjin GreenBio Materials Co. Ltd

(China), and Shenzhen Ecomann Technology Co. Ltd (China). According to the report "Polyhydroxyalkanoate (PHA) Market, By Application (Packaging, Food Services, Bio-medical, Agriculture) & Raw Material - Global Tends & Forecasts to 2018", they forecasted that PHA market consumption will reach 34,000 MT by 2018.

It was easily seen that basic research has tremendously contributed to enhance both the properties of the polymer as well as the productivity so that these polymers can eventually join the market, and serve society. As reviewed in the Chapter 1, P(3HB) was first investigated in a number of different bacteria. Even though this type of polymer is naturally produced in many bacteria, scientists still employ molecular tools, such as gene knock-out or heterologous expression, to engineer these strains so that it can produce the polymer even better with a higher yield. Based on these research efforts, it eventually came out into the market when ICI first launched it in the 1980s. However, P(3HB) is too brittle to compete with conventional plastics, resulting in endless efforts to search for alternative materials, such as P(3HB-*co*-3HV), MCL PHA, (SCL-*co*-MCL) PHA, PLA, P(3HB-*co*-LA) and so on. P(3HB-*co*-3HV) with a trade name Biopol was the second commercialized products made by Monsanto company. Therefore, basic research plays a critical role in gaining insights into different types of polymers and their properties to search for the best candidate as well as enhance the productivity to reduce the production cost. Because of their wide range of applications, the increase in industrial investment into PHA production is going to happen in the not too distant future.

With the same goal, our study has made advances in the exploitation of bacterial systems as efficient platforms for novel polymer production. We have shown that we can achieve a wide range of different types of polymers produced in *S. meliloti* and *P. putida* such as P(3HB), PLA, P(3HB-*co*-LA) and P(3HB-*co*-LA-*co*-3HHx-*co*-3HO). However, the production has not yet met

industrial requirements in terms of productivity and cost. Further studies are required to enhance the productivity so that these biopolymers may eventually be commercialized and mass-produced.

Appendix

A. Media recipes and reagent solutions

A.1 Media recipes

- Trypton Yeast (TY)/ 1L

Tryptone	5g
Yeast Extract	3g
$CaCl_2.2H_2O$	0.44g

- Luria-Bertani (LB)/ 1L

NaCl	5g
Tryptone	10g
Yeast Extract	5g

- M9 (20X stock)/ 1L

Na_2HPO_4	11.6g
KH_2PO_4	6g
NaCl	1g
NH_4Cl	2g

- 0.5E2/ 1L

NaNH4HPO4.4H2O	1.75g

K2HPO4.3H20	3.75g
KH2PO4	1.85g
MgSO4.7H2O (stock 100mM)	10ml
TMS	1ml

Trace metal solution (TMS): 2.78g $FeSO_4.7H_2O$, 1.98g $MnCl_2.4H_2O$, 2.81g $CoSO_4.7H_2O$, 1.47g $CaCl_2.2H_2O$, 0.17g $CuCl_2.2H_2O$, 0.29g $ZnSO_4.7H_2O$. in 1N HCl.

- Yeast Extract Mannitol (YEM)/ 1L

Yeast Extract	0.5g
K_2HPO_4	1g
NaCl	0.1g
$MgSO_4$	0.2g
$CaCl_2$	0.5g
$FeCl_3$	6 mL of $FeCl_3$ stock (0.67 g/L)

A.2 Reagent solution

- Reagents for plasmid miniprep/ gel purification

Solution 1: 50mM Tris, 10mM EDTA, 100 µg ml RNAse, pH8

Solution 2: 1% SDS, 0.2 M NaOH

Solution 3: 4 M guanidine, 0.9M potassium acetate, pH4.8

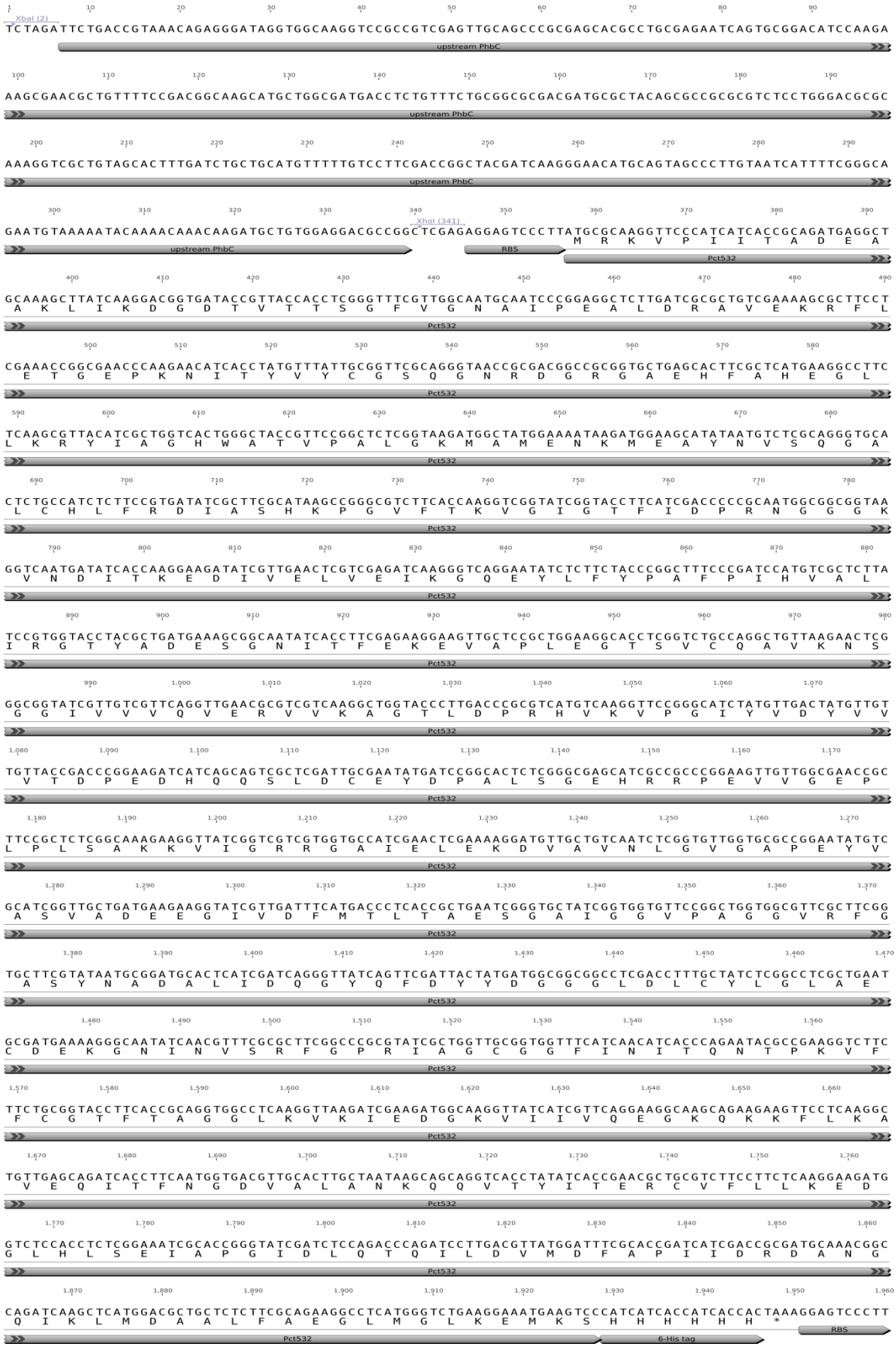
Elution: 2mM Tris

Binding buffer : 5.3M GUITC, 15mM EDTA, 120mM MFS,

- Reagent for enzyme assay

Sonication buffer 20mM TrisHcl, 1mM $MgCl_2$, 10% glycerol, 10mM β-mercapethanol

B. DNA sequence of synthesized genes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upstream PhbC
upstream PhbC
upstream PhbC
XhoI (341)
upstream PhbC
RBS
Pct532
Pct532
Pct532
Pct532
Pct532
Pct532
Pct532
Pct532
Pct532
Pct532
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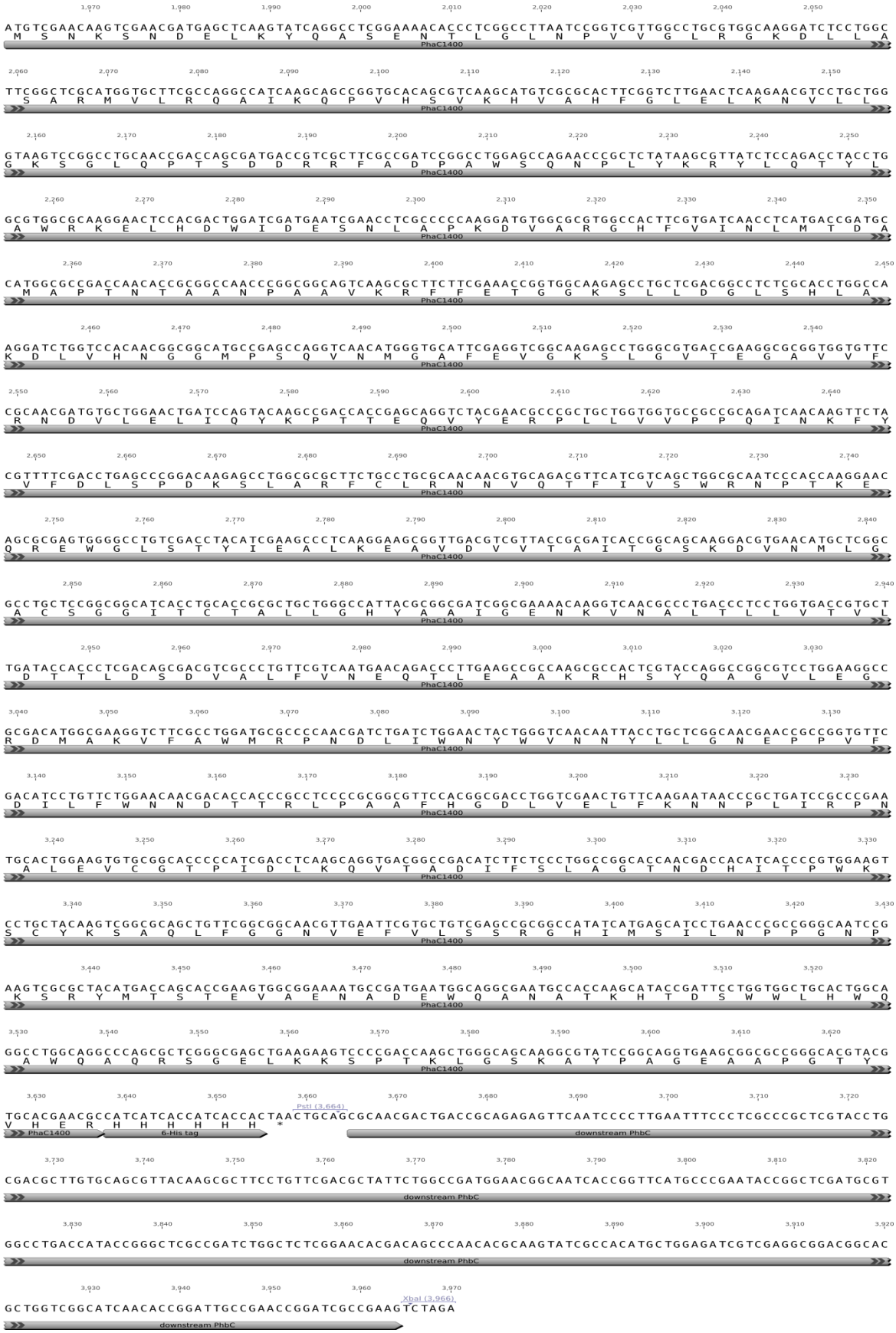
Pct532
Pct532
Pct532
Pct532
Pct532
RBS
Pct532
6-His tag


1,970 1,980 1,990 2,000 2,010 2,020 2,030 2,040 2,050
ATGTCGAACAAGTCGAACGATGAGCTCAAGTATCAGGCCTCGGAAAACACCCTCGGCCTTAATCCGGTCGTTGGCCTGCGTGGCAAGGATCTCCTGGC
M S N K S N D E L K Y Q A S E N T L G L N P V V G L R G K D L L A
PhaC1400
2,060 2,070 2,080 2,090 2,100 2,110 2,120 2,130 2,140 2,150
TTCGGCTCGCATGGTGCTTCGCCAGGCCATCAAGCAGCCGGTGCACAGCGTCAAGCATGTCGCGCACTTCGGTCTTGAACTCAAGAACGTCCTGCTGG
S A R M V L R Q A I K Q P V H S V K H V A H F G L E L K N V L L
PhaC1400
2,160 2,170 2,180 2,190 2,200 2,210 2,220 2,230 2,240 2,250
GTAAGTCCGGCCTGCAACCGACCAGCGATGACCGTCGCTTCGCCGATCCGGCCTGGAGCCAGAACCCGCTCTATAAGCGTTATCTCCAGACCTACCTG
G K S G L Q P T S D D R R F A D P A W S Q N P L Y K R Y L Q T Y L
PhaC1400
2,260 2,270 2,280 2,290 2,300 2,310 2,320 2,330 2,340 2,350
GCGTGGCGCAAGGAACTCCACGACTGGATCGATGAATCGAACCTCGCCCCCAAGGATGTGGCGCGTGGCCACTTCGTGATCAACCTCATGACCGATGC
A W R K E L H D W I D E S N L A P K D V A R G H F V I N L M T D A
PhaC1400
2,360 2,370 2,380 2,390 2,400 2,410 2,420 2,430 2,440 2,450
CATGGCGCCGACCAACACCGCGGCCAACCCGGCGGCAGTCAAGCGCTTCTTCGAAACCGGTGGCAAGAGCCTGCTCGACGGCCTCTCGCACCTGGCCA
M A P T N T A A N P A A V K R F F E T G G K S L L D G L S H L A
PhaC1400
2,460 2,470 2,480 2,490 2,500 2,510 2,520 2,530 2,540
AGGATCTGGTCCACAACGGCGGCATGCCGAGCCAGGTCAACATGGGTGCATTCGAGGTCGGCAAGAGCCTGGGCGTGACCGAAGGCGCGGTGGTGTTC
K D L V H N G G M P S Q V N M G A F E V G K S L G V T E G A V V F
PhaC1400
2,550 2,560 2,570 2,580 2,590 2,600 2,610 2,620 2,630 2,640
CGCAACGATGTGCTGGAACTGATCCAGTACAAGCCGACCACCGAGCAGGTCTACGAACGCCCGCTGCTGGTGGTGCCGCCGCAGATCAACAAGTTCTA
R N D V L E L I Q Y K P T T E Q V Y E R P L L V V P P Q I N K F Y
PhaC1400
2,650 2,660 2,670 2,680 2,690 2,700 2,710 2,720 2,730 2,740
CGTTTTCGACCTGAGCCCGGACAAGAGCCTGGCGCGCTTCTGCCTGCGCAACAACGTGCAGACGTTCATCGTCAGCTGGCGCAATCCCACCAAGGAAC
V F D L S P D K S L A R F C L R N N V Q T F I V S W R N P T K E
PhaC1400
2,750 2,760 2,770 2,780 2,790 2,800 2,810 2,820 2,830 2,840
AGCGCGAGTGGGGCCTGTCGACCTACATCGAAGCCCTCAAGGAAGCGGTTGACGTCGTTACCGCGATCACCGGCAGCAAGGACGTGAACATGCTCGGC
Q R E W G L S T Y I E A L K E A V D V V T A I T G S K D V N M L G
PhaC1400
2,850 2,860 2,870 2,880 2,890 2,900 2,910 2,920 2,930 2,940
GCCTGCTCCGGCGGCATCACCTGCACCGCGCTGCTGGGCCATTACGCGGCGATCGGCGAAAACAAGGTCAACGCCCTGACCCTCCTGGTGACCGTGCT
A C S G G I T C T A L L G H Y A A I G E N K V N A L T L L V T V L
PhaC1400
2,950 2,960 2,970 2,980 2,990 3,000 3,010 3,020 3,030
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D T T L D S D V A L F V N E Q T L E A A K R H S Y Q A G V L E G
PhaC1400
3,040 3,050 3,060 3,070 3,080 3,090 3,100 3,110 3,120 3,130
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R D M A K V F A W M R P N D L I W N Y W V N N Y L L G N E P P V F
PhaC1400
3,140 3,150 3,160 3,170 3,180 3,190 3,200 3,210 3,220 3,230
GACATCCTGTTCTGGAACAACGACACCACCCGCCTCCCCGCGGCGTTCCACGGCGACCTGGTCGAACTGTTCAAGAATAACCCGCTGATCCGCCCGAA
D I L F W N N D T T R L P A A F H G D L V E L F K N N P L I R P N
PhaC1400
3,240 3,250 3,260 3,270 3,280 3,290 3,300 3,310 3,320 3,330
TGCACTGGAAGTGTGCGGCACCCCCATCGACCTCAAGCAGGTGACGGCCGACATCTTCTCCCTGGCCGGCACCAACGACCACATCACCCCGTGGAAGT
A L E V C G T P I D L K Q V T A D I F S L A G T N D H I T P W K
PhaC1400
3,340 3,350 3,360 3,370 3,380 3,390 3,400 3,410 3,420 3,430
CCTGCTACAAGTCGGCGCAGCTGTTCGGCGGCAACGTTGAATTCGTGCTGTCGAGCCGCGGCCATATCATGAGCATCCTGAACCCGCCGGGCAATCCG
S C Y K S A Q L F G G N V E F V L S S R G H I M S I L N P P G N P
PhaC1400
3,440 3,450 3,460 3,470 3,480 3,490 3,500 3,510 3,520
AAGTCGCGCTACATGACCAGCACCGAAGTGGCGGAAAATGCCGATGAATGGCAGGCGAATGCCACCAAGCATACCGATTCCTGGTGGCTGCACTGGCA
K S R Y M T S T E V A E N A D E W Q A N A T K H T D S W W L H W Q
PhaC1400
3,530 3,540 3,550 3,560 3,570 3,580 3,590 3,600 3,610 3,620
GGCCTGGCAGGCCCAGCGCTCGGGCGAGCTGAAGAAGTCCCCGACCAAGCTGGGCAGCAAGGCGTATCCGGCAGGTGAAGCGGCGCCGGGCACGTACG
A W Q A Q R S G E L K K S P T K L G S K A Y P A G E A A P G T Y
PhaC1400
3,630 3,640 3,650 3,660 3,670 3,680 3,690 3,700 3,710 3,720
PstI (3,664)
TGCACGAACGCCATCATCACCATCACCACTAACTGCAGCGCAACGACTGACCGCAGAGAGTTCAATCCCCTTGAATTTCCCTCGCCCGCTCGTACCTG
V H E R H H H H H H *
PhaC1400
6-His tag
downstream PhbC
3,730 3,740 3,750 3,760 3,770 3,780 3,790 3,800 3,810 3,820
CGACGCTTGTGCAGCGTTACAAGCGCTTCCTGTTCGACGCTATTCTGGCCGATGGAACGGCAATCACCGGTTCATGCCCGAATACCGGCTCGATGCGT
downstream PhbC
3,830 3,840 3,850 3,860 3,870 3,880 3,890 3,900 3,910 3,920
GGCCTGACCATACCGGGCTCGCCGATCTGGCTCTCGGAACACGACAGCCCAACACGCAAGTATCGCCACATGCTGGAGATCGTCGAGGCGGACGGCAC
downstream PhbC
3,930 3,940 3,950 3,960 3,970
XbaI (3,966)
GCTGGTCGGCATCAACACCGGATTGCCGAACCGGATCGCCGAAGTCTAGA
downstream PhbC

C. Increasing the flow toward LA monomer fraction

C.1 Introduction

This strategy is to aim at increasing the flow toward producing more precursors of lactic acid monomesr derived from sugar. Two genes encoding the lactate dehydrogenase and the acetyl-CoA synthetase were selected and introduced into the engineered *S. meliloti* system in this study.

Lactate dehydrogenase links metabolites from the glycolysis pathway to the lactyl-CoA production by converting pyruvate into D-lactate as shown in Figure 3.1. Two genes encoding the lactate dehydrogenase were chosen to examine originated from *E. coli* and *S. fredii* strains. This NADH-linked enzyme encoded by the *ldhA* gene from *E. coli* strain has been well-characterised in previous studies (Bunch et al., 1997; Mat-jan et al., 1989). This gene was highly homologous to genes for other D-lactate-specific dehydrogenase but not to those for the L-lactate-specific enzymes. In contrast, the *glcD* gene (NGR_c2063) from *S. fredii* has not been studied well in the literature.

The acetyl CoA synthetase can metabolize acetate to acetyl CoA to supply the pool of CoA for precursors of polymer production. Two genes encoding the acetyl CoA synthetase were chosen to examine originated from *E. coli and S. fredii* strains. The *acs* gene from *E. coli* has been studied quite well previously while the *acs gene* from *S. fredii* strain has not been characterized (Barak and Abouhamad, 1998; Kumari et al., 2000).

C.2 Results and discussion

C.2.1 Construction of plasmids carrying genes from either *E. coli* or *S. meliloti*

The *ldhA* gene from *E. coli* strain was digested and introduced into the plasmid pSRKGm at NdeI and SacI sites to generate the new plasmid pSRK*ldhA*. Then, the *acs* gene from *E. coli* strain was digested and introduced into the plasmid pSRK*ldhA* at SacI and SpeI sites to generate the resulting plasmid pSRK*ldhAacs* as shown in Figure C.1.

The *glcD* gene from *S. fredii* strain was digested and introduced into the plasmid pSRKGm at NdeI and SpeI sites to generate the new plasmid pSRK*glcD*. Then, the *acs* gene from *S. fredii* strain was digested and introduced into the plasmid pSRK*glcD* at SpeI and HindIII sites to generate the resulting plasmid pSRK*glcDacs* as shown in Figure C.2.

These resulting plasmids were then introduced into engineered strains, SmUW254 and SmUW256 to examine the enzyme activities of the lactate dehydrogenases. In addition, in order to investigate the activity of the acetyl-CoA synthetase by observing the complementation of the *acs* mutant *S. meliloti* strain harbouring one of two constructed plasmids (pSRK*ldhAacs* or pSRK*glcDacs*).

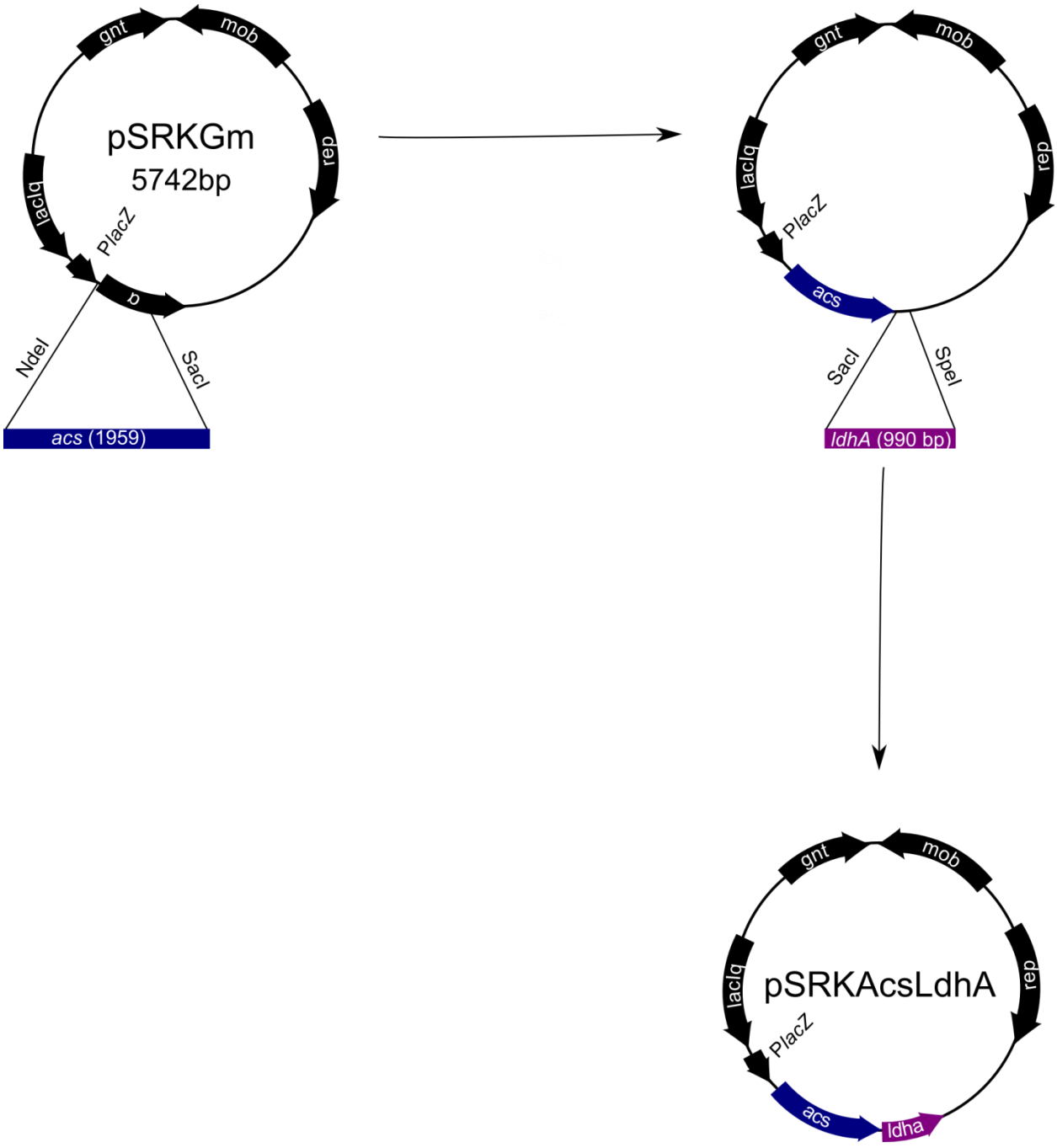

Figure C.1 Construction of expression plasmid pSRK*acsldhA*. Genes including *acs* and *ldhA* from *E. coli* were introduced into pSRKGm as outlined in the above figure.

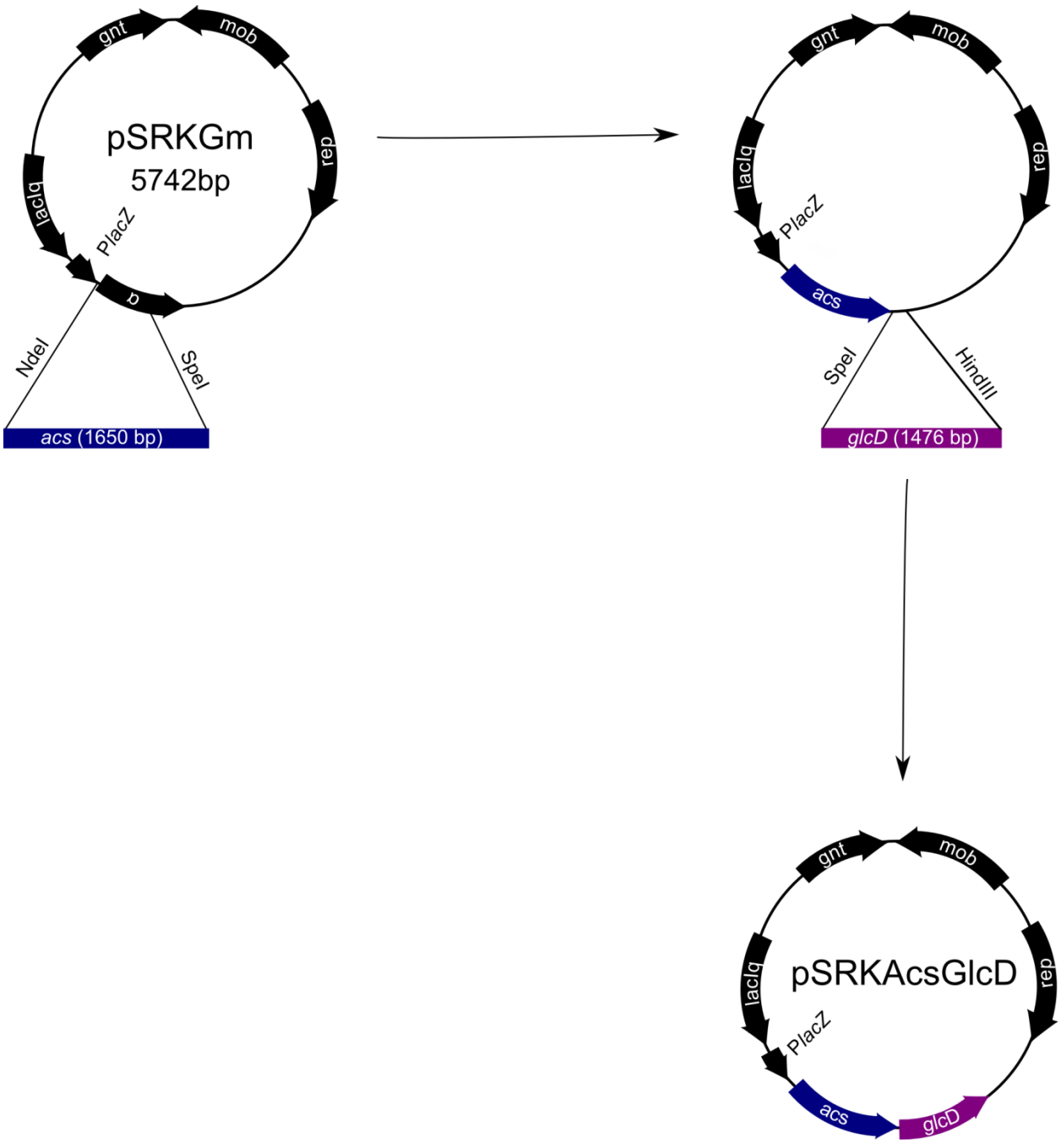

Figure C.2 Construction of expression plasmid pSRK*acsglcD*. Genes including *acs* and *glcD* from *S. fredii* were introduced into pSRKGm as outlined in the above figure.

C.2.2 Measurement of lactate dehydrogenase activity

Introducing the pSRK*acsldhA* and pSRK*acsglcD* into engineered strains (SmUW254 or SmUW256) by conjugation. Then enzyme assays were performed to determine the activity of the lactate dehydrogenase.

For the chromosome-engineered strain SmUW254, the assays were performed with the strains equipped with the lactate dehydrogenase gene from either *E. coli* or *S. meliloti* under inducing and non-inducing conditions. Only the lactate dehydrogenase gene from *S. fredii* showed the increase in lactate dehydrogenase activity as shown in Figure C.3. It could be that the gene from *E. coli* was unable to be expressed in *S. meliloti* because the codon usage in *E. coli* is different from that in *S. meliloti.*

For the plasmid-based engineered strain SmUW256, the assays were also performed with the strains equipped with the lactate dehydrogenase gene from either *E. coli* or *S. meliloti* under inducing and non-inducing conditions. However, the enzyme activity was unable to detected regardless of which lactate dehygrogenase gene was used. The reason still remained unknown; however, using two inducible plasmids in the same strain might have some drawbacks for expression system.

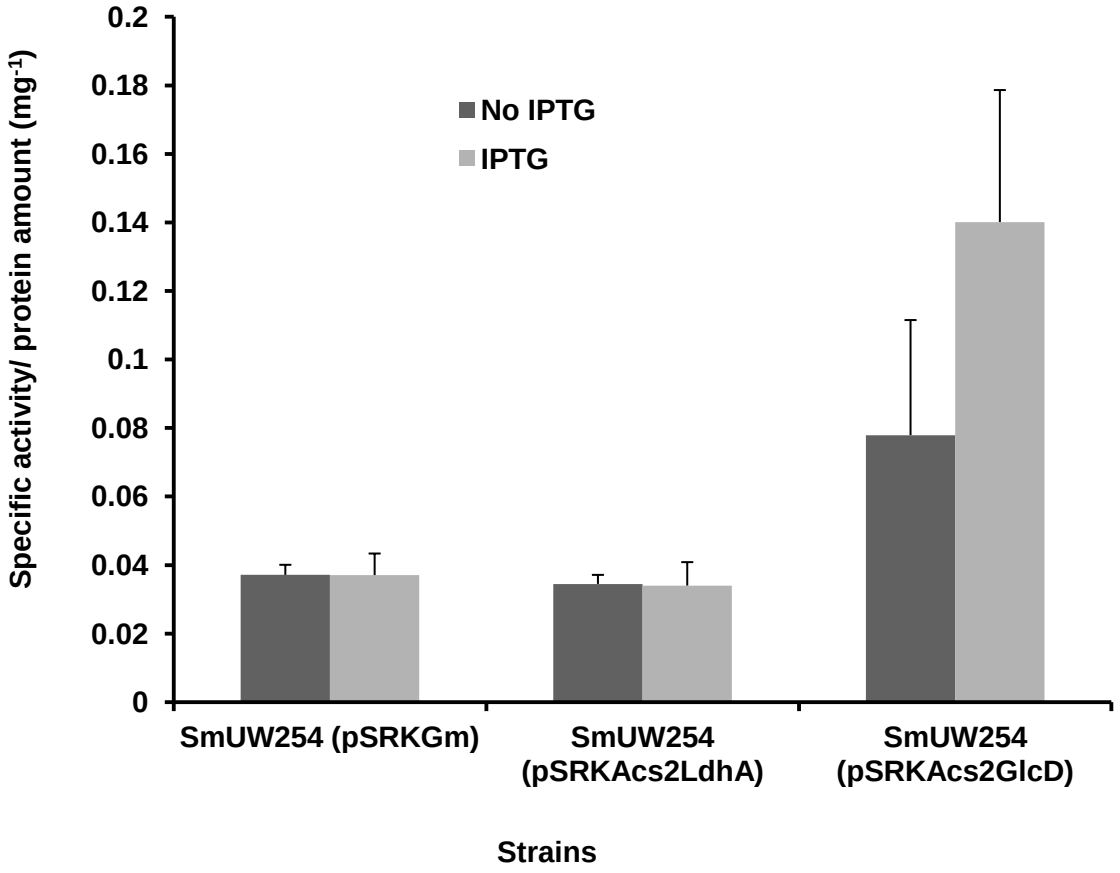

Figure C.3 Measurement of lactate dehydrogenase activity when expressing *ldhA* gene from *E. coli* and *glcD* gene from *S. fredii* in *S. meliloti* SmUW254 background in both inducing and non-inducing conditions.

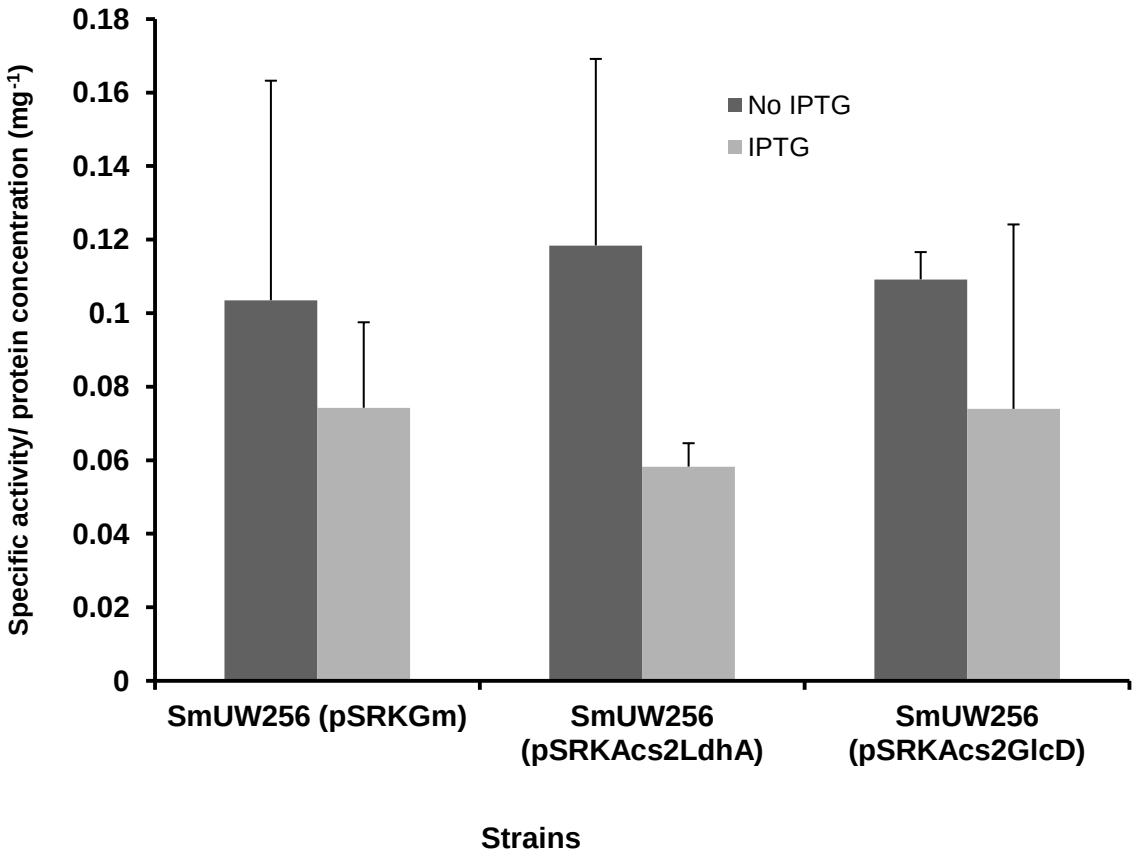

Figure C.4 Measurement of lactate dehydrogenase activity when expressing *ldhA* gene from *E. coli* and *glcD* gene from *S. fredii* in *S. meliloti* SmUW256 background in both inducing and non-inducing conditions.

C.2.3 Measurement of acetyl-CoA synthetase activity

To investigate the enzyme activity of acetyl-CoA synthetase, genes from *E. coli* and *S. fredii* were introduced and expressed in *acs* mutant *S. meliloti* strain Rm11382. Then, the growth curve was constructed by recording the absorbance at 600nm over time (Figure C.5). The wild-type strain Rm1021 was used as a positive control; the *acs* mutant *S. meliloti* harbouring the empty plasmid was used as a negative control. Only the strain carrying the gene from *E. coli* was able to grow in M9 media supplemented. The growth of Rm is quite slow compared to that of the wild-type strain.

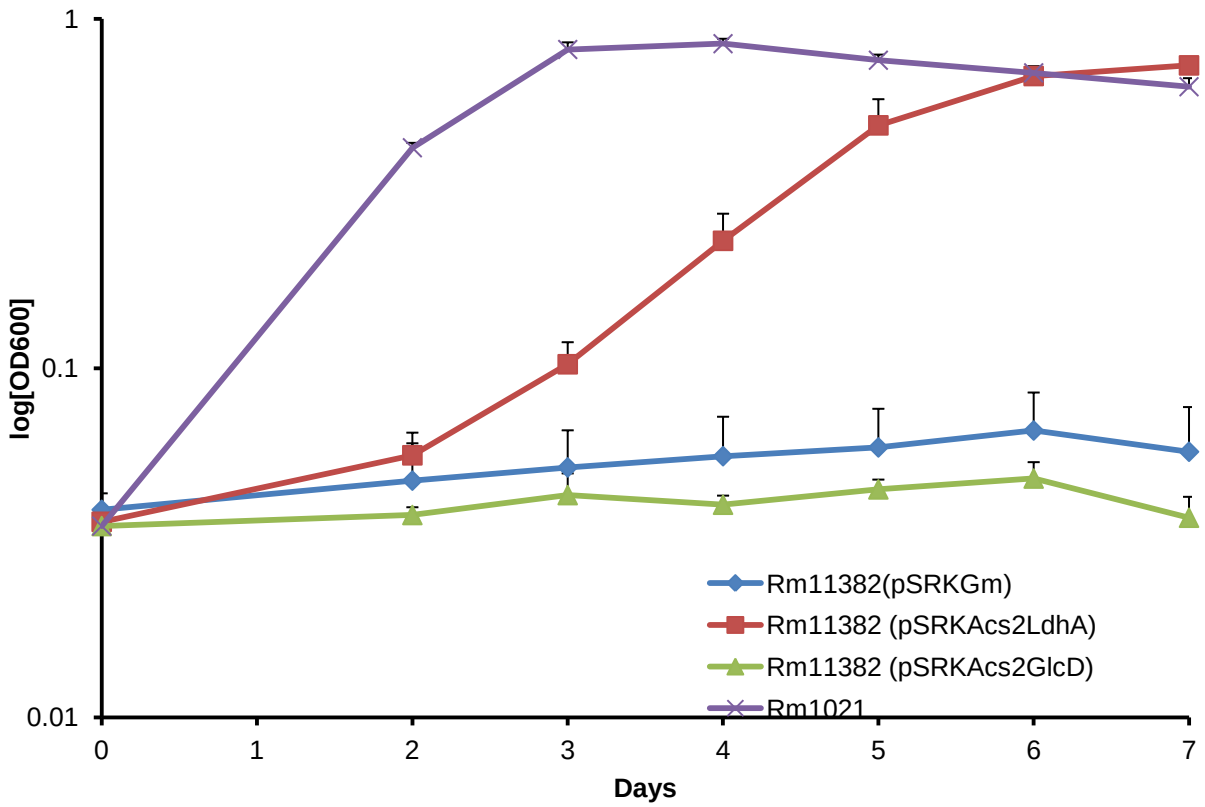

Figure C.5 Growth curve of different strains on M9 supplemented with acetate as sole carbon source.

D. Other data

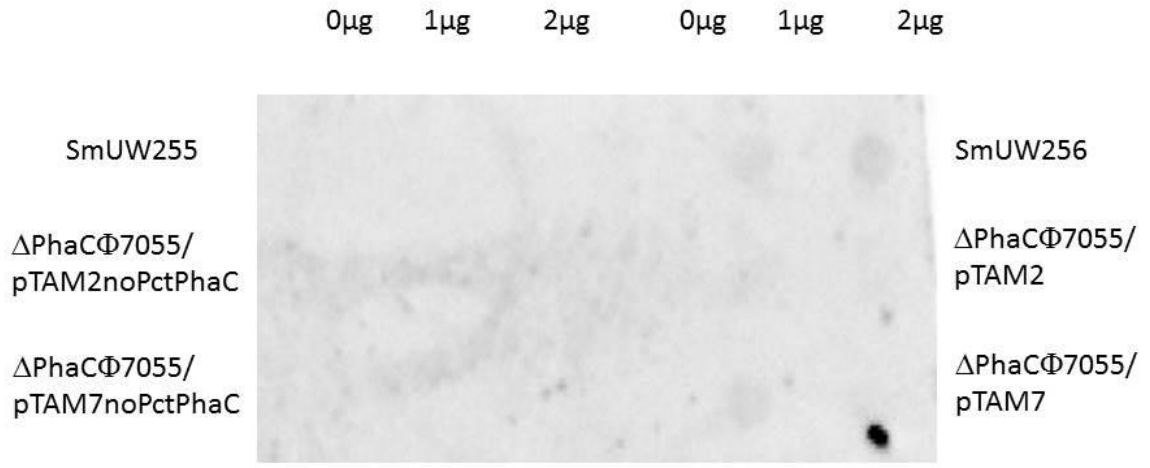

Figure D.1 Dot blot image of *S. meliloti* SmUW499 carrying plasmids of different promoters using *pct* probe. Three left columns represent RNA samples of different amounts (0, 1, 2µg) of SmUW255 (SmUW499 (pTH1227)), SmUW564 (SmUW499 (pTAM2noinsert)), SmUW567 (SmUW499 (pTAM7noinsert)). The other right three columns represent RNA samples of different amounts (0, 1, 2µg) of SmUW256 (SmUW499 (pTAM)), SmUW558 (SmUW499 (pTAM2)), SmUW563 (SmUW499 (pTAM7)).

Bibliography

Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2006. Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U. S. A.* **103**:12678–12683.

Amann E, Ptaslme M. 1983. Vectors bearing a hybrid trp-lac promoter useful for regulated expression of doned genes in Escherichia coil. *Gene* **25**:167–178.

Amass W, Amass A, Tighe B. 1998. A Review of Biodegradable Polymers : Uses , Current Developments in the Synthesis and Characterization of Biodegradable Polyesters , Blends of Biodegradable Polymers and Recent Advances in Biodegradation Studies. *Polym. Int.* **47**:89–144.

Anderson a J, Dawes E a. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54**:450–72.

Aneja P, Dai M, Lacorre DA, Pillon B, Charles TC. 2004. Heterologous complementation of the exopolysaccharide synthesis and carbon utilization phenotypes of Sinorhizobium meliloti Rm1021 polyhydroxyalkanoate synthesis mutants. *FEMS Microbiol. Lett.* **239**:277–283.

Barak R, Abouhamad WN. 1998. Both Acetate Kinase and Acetyl Coenzyme A Synthetase Are Involved in Acetate-Stimulated Change in the Direction of Flagellar Rotation in Escherichia coli. *J. Bateriology* **180**:985–988.

Bélanger L, Dimmick KA, Fleming JS, Charles TC. 2009a. Null mutations in Sinorhizobium meliloti exoS and chvI demonstrate the importance of this two-component regulatory system for symbiosis. *Mol. Microbiol.* **74**:1223–1237.

Bélanger L, Dimmick K a, Fleming JS, Charles TC. 2009b. Null mutations in Sinorhizobium meliloti exoS and chvI demonstrate the importance of this two-component regulatory system for symbiosis. *Mol. Microbiol.* **74**:1223–37.

Bernd H. A. Rehm NK, ¨ ger and AS, Chel ¨. 1998. A New Metabolic Link between Fatty Acid de Novo Synthesis and Polyhydroxyalkanoic Acid Synthesis. *J. Biol. Chem.* **273**:24044 – 24051.

Birnboim H., Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1524.

Blazeck J, Alper HS. 2013. Promoter engineering: recent advances in controlling transcription at the most fundamental level. *Biotechnol. J.* **8**:46–58.

De Boer HA, Comstock LJ, Vasser M. 1983. The tac promoter: A functional hybrid derived from the trp and lac promoters. *Proc. Natl. Acad. Sci. U. S. A.* **80**:21–25.

Bohmert K, Balbo I, Kopka J, Mittendorf V, Nawrath C, Poirier Y, Tischendorf G, Trethewey RN, Willmitzer L. 2000. Transgenic Arabidopsis plants can accumulate polyhydroxybutyrate to up to 4 % of their fresh weight. *Planta* **211**:841–845.

Borrero-de Acuña JM, Bielecka A, Häussler S, Schobert M, Jahn M, Wittmann C, Jahn D, Poblete-Castro I. 2014. Production of medium chain length polyhydroxyalkanoate in metabolic flux optimized Pseudomonas putida. *Microb. Cell Fact.* **13**:1–15.

Braunegg G, Bogensberger B. 1986. Zur Kinetik des Wachstums und der Speicherung von Poly-D(-) -3-hydroxybuttersaure bei Alcaligenes latus. *Acta Biotechnol.* **6**:339–345.

Breuer U, Terentiev Y, Kunze G, Babel W. 2002. Yeasts as Producers of Polyhydroxyalkanoates: Genetic Engineering of Saccharomyces cerevisiae. *Macromol. Biosci.* **2**:380–386.

Bunch PK, Mat-jan F, Lee N, Clark DP. 1997. The ldhA gene encoding the fermentative lactate dehydrogenase of Escherichia coli. *Enzyme* **143**:187–195.

Byrom D. 1987. Polymer synthesis by micro- organisms: technology and economics. *Trends Biotechnol.* **5**:246–250.

Calos MP. 1978. DNA sequence for a low-level promoter of the lac repressor gene and an “up” promoter mutation. *Nature* **274**:762–765.

Chan CL, Gross C a. 2001. The anti-initial transcribed sequence, a portable sequence that impedes promoter escape, requires sigma70 for function. *J. Biol. Chem.* **276**:38201–38209.

Charles TC, Cai G, Aneja P. 1997. Megaplasmid and chromosomal loci for the PHB degradation pathway in Rhizobium (Sinorhizobium) meliloti. *Genetics*:1211–1220.

Charles TC, Finan TM. 1990. Genetic Map of Rhizobium meliloti Megaplasmid pRmeSU47b. *J. Bacteriol.* **172**:2469–2476.

Charles TC, Finan TM. 1991. Analysis of a 1600-Kilobase Rhizobium meliloti Megaplasmid Using Defined Deletions Generated in Vivo. *Genetics* **127**:5–20.

Cheng J, Sibley CD, Zaheer R, Finan TM. 2007. A Sinorhizobium meliloti minE mutant has an altered morphology and exhibits defects in legume symbiosis Printed in Great Britain. *Microbiology*:375–387.

Choi JI, Lee SY, Han K. 1998. Cloning of the Alcaligenes latus polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of Poly(3-hydroxybutyrate) in Escherichia coli. *Appl. Environ. Microbiol.* **64**:4897–903.

Choi J, Lee SY. 1999. High-Level Production of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) by Fed-Batch Culture of Recombinant Escherichia coli. *Appl. Environ. Microbiol.* **65**:4364–4368.

D'Haeze W, Gao M, Holsters M. 2004. A gfp reporter plasmid to visualize Azorhizobium caulinodans during nodulation of Sesbania rostrata. *Plasmid* **51**:185–191.

Daae EB, Dunnill P, Mitsky T a, Padgette SR, Taylor NB, Valentin HE, Gruys KJ. 1999. Metabolic modeling as a tool for evaluating polyhydroxyalkanoate copolymer production in plants. *Metab. Eng.* **1**:243–254.

Davis JH, Rubin AJ, Sauer RT. 2011. Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Res.* **39**:1131–1141.

Doi Y, Kawaguchi Y, Koyama N, Nakamura S, Hiramitsu M. 1992. Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus. *FEMS Microbiol. Rev.* **103**:103–108.

Doi Y, Kitamura S, Abe H. 1995. Microbial Synthesis and Characterization of Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromolecules*:4822–4828.

Finan TM, Kunkel B, Vos GFDE, Signer ER. 1986. Second Symbiotic Megaplasmid in Rhizobium meliloti Carrying Exopolysaccharide and Thiamine Synthesis Genes. *J. Bacteriol.* **167**:66–72.

Fritzsche K, Lenz RW, Fuller RC. 1990. Production of unsaturated polyesters by Pseudomonas oleovorans. *Int. J. Biol. Macromol.* **12**:85–91.

Fukui T, Doi Y. 1997. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of Aeromonas caviae. *J. Bacteriol.* **179**:4821–4830.

Fukui T, Shiomi N, Doi Y. 1998. Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by Aeromonas caviae. *J. Bacteriol.* **180**:667–673.

Fukui T, Yokomizo S, Kobayashi G, Doi Y. 1999. Co-expression of polyhydroxyalkanoate synthase and (R)-enoyl-CoA hydratase genes of Aeromonas caviae establishes copolyester biosynthesis pathway in Escherichia coli. *FEMS Microbiol. Lett.* **170**:69–75.

Fukui T, Abe H, Doi Y. 2002. Engineering of Ralstonia eutropha for Production of Poly(3-hydroxybutyrate- co -3-hydroxyhexanoate) from Fructose and Solid-State Properties of the Copolymer. *Biomacromolecules* **3**:618–624.

Genser KF, Renner G, Schwab H. 1998. Molecular cloning, sequencing and expression in Escherichia coli of the poly(3-hydroxyalkanoate) synthesis genes from Alcaligenes latus DSM1124. *J. Biotechnol.* **64**:125–135.

Green PR, Kemper J, Schechtman L, Guo L, Satkowski M, Fiedler S, Steinbüchel A, Rehm BH a. 2002. Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid beta-oxidation inhibited Ralstonia eutropha. *Biomacromolecules* **3**:208–213.

Gross R, Kalra B, Kumar A. 2001. Polyester and polycarbonate synthesis by in vitro enzyme catalysis. *J. Polym. Sci.*:655–660.

Gross RA, Demello C, Lenz RW. 1989. Biosynthesis and Characterization of Poly(β-hydroxyalkanoates) Produced by Pseudomonas oleovorans. *Macromolecules* **22**:1106–1115.

Grote A, Hiller K, Scheer M, Munch R, Nortemann B, Hempel DC, Jahn D. 2005. JCat : a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* **33**:526–531.

Gurich N, González JE. 2009. Role of quorum sensing in Sinorhizobium meliloti-Alfalfa symbiosis. *J. Bacteriol.* **191**:4372–4382.

Hammer K, Mijakovic I, Jensen PR. 2006. Synthetic promoter libraries--tuning of gene expression. *Trends Biotechnol.* **24**:53–55.

Haynes D, Abayasinghe NK, Harrison GM, Burg KJ, Smith DW. 2007. In Situ Copolyesters Containing Poly(L-lactide) and Poly(hydroxyalkanoate) Units. *Biomacromolecules* **8**:1131–1137.

Heil JR, Nordeste RF, Charles TC. 2011. The Fluorescence Theatre : a cost-effective device using theatre gels for fluorescent protein and dye screening. *Can. J. Microbiol.* **342**:339–342.

Hernandez VJ, Hsu LM, Cashel M. 1996. Conserved Region 3 of Escherichia coli 70 Is Implicated in the Process of Abortive Transcription. *J. Biol. Chem.* **271**:18775–18779.

Hiraishi A, Khan ST. 2003. Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. *Appl. Microbiol. Biotechnol.* **61**:103–109.

Hoffmann N, Steinbüchel A, Rehm BH. 2000. Homologous functional expression of cryptic phaG from Pseudomonas oleovorans establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. *Appl. Microbiol. Biotechnol.* **54**:665–70.

Hoffmann N, Amara A a, Beermann BB, Qi Q, Hinz H-J, Rehm BH a. 2002. Biochemical characterization of the Pseudomonas putida 3-hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. *J. Biol. Chem.* **277**:42926–36.

Hoffmann N, Rehm BH a. 2004a. Regulation of polyhydroxyalkanoate biosynthesis in Pseudomonas putida and Pseudomonas aeruginosa. *FEMS Microbiol. Rev.* **237**:1–7.

Hoffmann N, Rehm BH a. 2004b. Regulation of polyhydroxyalkanoate biosynthesis in Pseudomonas putida and Pseudomonas aeruginosa. *FEMS Microbiol. Rev.* **237**:1–7.

Holmes PA. 1985. Applications of PHB - A Microbially Produced Biodegradable Thermoplastic. *Physic Technol.* **16**:32–36.

Hrabak O. 1992. Industrial production of poly-β-hydroxybutyrate. *FEMS Microbiol. Rev.* **103**:251–255.

Huijberts GN, Eggink G, de Waard P, Huisman GW, Witholt B. 1992. Pseudomonas putida KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. *Appl. Environ. Microbiol.* **58**:536–544.

Huijberts GN, de Rijk TC, de Waard P, Eggink G. 1994. 13C nuclear magnetic resonance studies of Pseudomonas putida fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *J. Bacteriol.* **176**:1661–1666.

Huisman GW, de Leeuw O, Eggink G, Witholt B. 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl. Environ. Microbiol.* **55**:1949–1954.

Huisman GW, Wonink E, Koning G De, Preusting H, Witholt B. 1992. Synthesis of poly (3-hydroxyalkanoates) by mutant and recombinant Pseudomonas strains. *Appl. Microbiol. Biotechnol.*:1–5.

Huisman GW, Wonink E, Meima R, Kazemier B, Terpstra P. 1991. Metabolism of Poly(3-hydroxyalkanoates) (PHAs) by Pseudomonas oleovorans. *J. Biol. Chem.* **266**:2191–2198.

Jensen PR, Hammer K. 1998. The Sequence of Spacers between the Consensus Sequences Modulates the Strength of Prokaryotic Promoters The Sequence of Spacers between the Consensus Sequences Modulates the Strength of Prokaryotic Promoters. *Appl. Environ. Microbiol.* **64**:82–87.

Jiang X, Ramsay J a, Ramsay B a. 2006. Acetone extraction of mcl-PHA from Pseudomonas putida KT2440. *J. Microbiol. Methods* **67**:212–209.

Jørgensen CM, Hammer K, Jensen PR, Martinussen J. 2004. Expression of the pyrG gene determines the pool sizes of CTP and dCTP in Lactococcus lactis. *Eur. J. Biochem.* **271**:2438–2345.

Jung Y, Lee S, Tran T. 2010a. Towards Systems Metabolic Engineering of PHA Producers. Ed. Guo-Qiang Chen Plastics f. Berlin Heidelberg: Springer-Verlag.

Jung YK, Kim TY, Park SJ, Lee SY. 2010b. Metabolic Engineering of Escherichia coli for the Production of Polylactic Acid and Its Copolymers. *Biotechnol. Bioeng.* **105**:161–171.

Kahar P, Agus J, Kikkawa Y, Taguchi K. 2005. Effective production and kinetic characterization of ulltra-high-molecular-weight poly[(R)-3-hydroxybutyrate] in recombinant Escherichia coli. *Polym. Degrad. Stab.* **87**:161–169.

Kammerer W, Deuschle U, Gentz R, Bujard H. 1986. Functional dissection of Escherichia coli promoters : information in the transcribed region is involved in late steps of the overall process. *EMBO J.* **5**:2995–3000.

Kelly JR, Rubin AJ, Davis JH, Ajo-Franklin CM, Cumbers J, Czar MJ, de Mora K, Glieberman AL, Monie DD, Endy D. 2009. Measuring the activity of BioBrick promoters using an in vivo reference standard. *J. Biol. Eng.* **3**:1–13.

Keren L, Zackay O, Lotan-Pompan M, Barenholz U, Dekel E, Sasson V, Aidelberg G, Bren A, Zeevi D, Weinberger A, Alon U, Milo R, Segal E. 2013. Promoters maintain their relative activity levels under different growth conditions. *Mol. Syst. Biol.* **9**:1–17.

Khan SR, Gaines J, Roop RM, Farrand SK. 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl. Environ. Microbiol.* **74**:5053–5062.

Klinke S, Ren Q, Witholt B, Kessler B. 1999. Production of medium-chain-length poly(3-hydroxyalkanoates) from gluconate by recombinant Escherichia coli. *Appl. Environ. Microbiol.* **65**:540–538.

Kolybaba M, Tabil LG, Panigrahi S, Crerar WJ, Powell T, Wang B, Inn Q, North A, Dakota N. 2003. Biodegradable Polymers : Past , Present , and Future. *CSAE/ASAE Annu. Intersect. Meet.* **0300**:1–15.

Koning G De. 1995. Physical properties of bacterial poly((R)-3-hydroxyal kanoates). *Can. J. Microbiol.* **41**:303–309.

Kumari S, Beatty CM, Browning DF, Busby SJW, Simel EJ, Hovel-Miner G, Wolfe a. J. 2000. Regulation of Acetyl Coenzyme A Synthetase in Escherichia coli. *J. Bacteriol.* **182**:4173–4179.

Langenbach S, Rehm BH a, Steinbüchel A. 1997. Functional expression of the PHA synthase gene phaC1 from Ps.pdf. *FEMS Microbiol. Lett.* **150**:303–309.

Lee SY, Chang HN. 1995. Production of poly(3-hydroxybutyric acid) by recombinant Escherichia coli strains: genetic and fermentation studies. *Can. J. Microbiol.* **41 Suppl 1**:207–215.

Lee SY, Wong HH, Choi JI, Lee SH, Lee SC, Han CS. 2000. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of Pseudomonas putida under phosphorus limitation. *Biotechnol. Bioeng.* **68**:466–470.

Lee SY. 1996. Review Bacterial Polyhydroxyalkanoates. *Biotechnol. Bioeng.* **49**:1–14.

Lee SJ, Park JP, Park TJ, Lee SY, Lee S, Park JK. 2005. Selective immobilization of fusion proteins on poly(hydroxyalkanoate) microbeads. *Anal. Chem.* **77**:5755–5749.

Leigh JA, Signer ER, Walker GC. 1985. Exopolysaccharide-deficient mutants of Rhizobium meliloti that form ineffective nodules. *Proc. Natl. Acad. Sci. U. S. A.* **82**:6231–6235.

Letouvet-Pawlak B, Monnier C, Barray S, Hodgson D., Guespin-Michel J. 1990. COMPARISON OF β-GALACTOSIDASE PRODUCTION BY TWO INDUCIBLE PROMOTERS IN MYXOCOCCUS XANTHUS. *Res. Microbiol.* **141**:425–435.

Leveau JHJ, Lindow SE. 2001. Predictive and Interpretive Simulation of Green Fluorescent Protein Expression in Reporter Bacteria Predictive and Interpretive Simulation of Green Fluorescent Protein Expression in Reporter Bacteria. *J. Bacteriol.* **183**:6752–6762.

Lin-Chao S, Bremer H. 1986. Effect of the bacterial growth rate on replication control of plasmid pBR322 in Escherichia coli. *Mol. Gen. Genet.* **1**:143–149.

Liu W, Chen G-Q. 2007. Production and characterization of medium-chain-length polyhydroxyalkanoate with high 3-hydroxytetradecanoate monomer content by fadB and fadA knockout mutant of Pseudomonas putida KT2442. *Appl. Microbiol. Biotechnol.* **76**:1153–1159.

Loo C-Y, Lee W-H, Tsuge T, Doi Y, Sudesh K. 2005. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3- hydroxyhexanoate) from palm oil products in a Wautersia eutropha mutant. *Biotechnol. Lett.* **27**:1405–1410.

Luengo JM, García B, Sandoval A, Naharro G, Olivera ER. 2003. Bioplastics from microorganisms. *Curr. Opin. Microbiol.* **6**:251–260.

Lunt J. 1998. Large-scale production, properties and commercial applications of polylactic acid polymers. *Polym. Degrad. Stab.* **3910**:145–152.

MacLellan SR, MacLean AM, Finan TM. 2006. Promoter prediction in the rhizobia. *Microbiology* **152**:1751–1763.

Marchesini S, Erard N, Glumoff T, Hiltunen JK, Poirier Y. 2003. Modification of the Monomer Composition of Polyhydroxyalkanoate Synthesized in Saccharomyces cerevisiae Expressing Variants of the β-Oxidation-Associated Multifunctional Enzyme. *Appl. Environ. Microbiol.* **69**:6495–6499.

Marsh JL, Erfle M, Wykes EJ. 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32**:481–485.

Martin L, Che A, Endy D. 2009. Gemini, a bifunctional enzymatic and fluorescent reporter of gene expression. *PLoS One* **4**:1–8.

Mat-jan F, Alam KY, Clark DP. 1989. Mutants of Escherichia coli Deficient in the Fermentative Lactate Dehydrogenase. *J. Bacteriol.* **171**:342–348.

McInerney MJ, Amos DA, Kealy KS, Palmer JA. 1992. Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. *FEMS Microbiol. Lett.* **103**:195–205.

Meade HM, Long SR, Ruvkun GB, Ausubel FM. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of Physical and Genetic Characterization of Symbiotic and Auxotrophic Mutants of Rhizobium meliloti Induced by Transposon TnS Mutagenesis. *J. Bacteriol.* **149**:114–122.

Meng W, Belyaeva T, Savery NJ, Busby SJW, Ross WE, Gaal T, Gourse RL, Thomas MS. 2001. UP element-dependent transcription at the Escherichia coli rrnB P1 promoter : positional requirements and role of the RNA polymerase α subunit linker. *Nucleic Acids Res.* **29**:4166–4178.

Le Meur S, Zinn M, Egli T, Thöny-Meyer L, Ren Q. 2012. Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered Pseudomonas putida KT2440. *BMC Biotechnol.* **12**:1–12.

Murphy KF, Balázsi G, Collins JJ. 2007. Combinatorial promoter design for engineering noisy gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **104**:12726–12731.

Myronovskyi M, Welle E, Fedorenko V, Luzhetskyy A. 2011. β-Glucuronidase as a Sensitive and Versatile Reporter in Actinomycetes. *Appl. Environ. Microbiol.* **77**:5370–5383.

Nawrath C, Poirier Y, Somervillet C. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. U. S. A.* **91**:12760–12764.

Nduko JM, Matsumoto K, Ooi T, Taguchi S. 2014. Enhanced production of poly(lactate-co-3-hydroxybutyrate) from xylose in engineered Escherichia coli overexpressing a galactitol transporter. *Appl. Microbiol. Biotechnol.* **98**:2453–60.

Noda I, Green PR, Satkowski MM, Schechtman L a. 2005. Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. *Biomacromolecules* **6**:580–576.

Noda I, Satkowski MM, Dowrey AE, Marcott C. 2004. Polymer alloys of Nodax copolymers and poly(lactic acid). *Macromol. Biosci.* **4**:269–275.

Nomura CT, Taguchi K, Taguchi S, Doi Y. 2004. Coexpression of Genetically Engineered 3-Ketoacyl-ACP Synthase III (fabH) and Polyhydroxyalkanoate Synthase (phaC) Genes

Leads to Copolymer Production from Glucose in Escherichia coli JM109. *Appl. Environ. Microbiol.* **70**:999–1007.

Okada M. 2002. Chemical syntheses of biodegradable polymers. *Prog. Polym. Sci.* **27**:87–133.

Oliveira VC De, Maeda I, Delessert S, Poirier Y. 2004. Increasing the Carbon Flux toward Synthesis of Short-Chain-Length −−Medium-Chain Length Polyhydroxyalkanoate in the Peroxisome of Saccharomyces cerevisiae through Modification of the β -Oxidation Cycle. *Appl. Environ. Microbiol.* **70**:1–4.

Ouyang S-P, Luo RC, Chen S-S, Liu Q, Chung A, Wu Q, Chen G-Q. 2007. Production of polyhydroxyalkanoates with high 3-hydroxydodecanoate monomer content by fadB and fadA knockout mutant of Pseudomonas putida KT2442. *Biomacromolecules* **8**:2504–2511.

Park SJ, Choi J, Lee SY. 2005a. Short-chain-length polyhydroxyalkanoates: Synthesis in metabolically engineered Escherichia.

Park SJ, Choi J, Lee SY. 2005b. Engineering of Escherichia coli fatty acid metabolism for the production of polyhydroxyalkanoates. *Enzyme Microb. Technol.* **36**:579–588.

Park SJ, Lee SY. 2003. Identification and Characterization of a New Enoyl Coenzyme A Hydratase Involved in Biosynthesis of Medium-Chain-Length Polyhydroxyalkanoates in Recombinant Escherichia coli. *J. Bacteriol.* **185**:5391–5397.

Park SJ, Lee SY. 2004. New FadB homologous enzymes and their use in enhanced biosynthesis of medium-chain-length polyhydroxyalkanoates in FadB mutant Escherichia coli. *Biotechnol. Bioeng.* **86**:681–6.

Park SJ, Park JP, Lee SY. 2002. Metabolic engineering of Escherichia coli for the production of medium-chain-length polyhydroxyalkanoates rich in specific monomers. *FEMS Microbiol. Lett.* **214**:217–22.

Park SJ, Park JP, Lee SY, Doi Y. 2003. Enrichment of specific monomer in medium-chain-length poly(3-hydroxyalkanoates) by amplification of fadD and fadE genes in recombinant Escherichia coli. *Enzyme Microb. Technol.* **33**:62–70.

Park TJ, Park JP, Lee SJ, Hong HJ, Lee SY. 2006. Polyhydroxyalkanoate chip for the specific immobilization of recombinant protein and its application in immunodiagnostics. *Biotechnol. Bioprocess Eng.* **11**:173–177.

Pauli G, Overath P. 1972. ato Operon: a Highly Inducible System for Acetoacetate and Butyrate Degradation in Escherichia coli. *Eur. J. Biochem.* **29**:553–562.

Peoples OP, Sinskey AJ. 1989. Poly-beta-hydroxybutyrate (PHB) Biosynthesis in Alcaligenes eutrophus H16. IDENTIFICATION AND CHARACTERIZATION OF THE PHB POLYMERASE GENE (phbC). *J. Biol. Chem.* **264**:15298–15303.

Peters V, Rehm BH a. 2006. In vivo enzyme immobilization by use of engineered polyhydroxyalkanoate synthase. *Appl. Environ. Microbiol.* **72**:1777–1783.

Pieper U, Steinbiichel A. 1992. Identification , cloning and sequence analysis of the poly (3-hydroxyalkanoic acid) synthase gene of the Gram-positive bacterium Rhodococcus ruber. *FEMS Microbiol. Lett.* **96**:73–80.

Poirier Y. 2002. Polyhydroxyalknoate synthesis in plants as a tool for biotechnology and basic studies of lipid metabolism. *Prog. Lipid Res.* **41**:131–155.

Poirier Y, Erard N, Pete JM. 2001. Synthesis of Polyhydroxyalkanoate in the Peroxisome of Saccharomyces cerevisiae by Using Intermediates of Fatty Acid β-Oxidation. *Appl. Environ. Microbiol.* **67**:5254–5260.

Prather K. 2010. Low-copy number plasmids as artificial chromosomes. In: Smolke, C, editor. *Metab. Pathw. Eng. Handb.* New York: CRC Press, pp. 1–17.

Qi Q, Rehm BH, Steinbüchel A. 1997. Synthesis of poly(3-hydroxyalkanoates) in Escherichia coli expressing the PHA synthase gene phaC2 from Pseudomonas aeruginosa: comparison of PhaC1 and PhaC2. *FEMS Microbiol. Lett.* **157**:155–162.

Reeve WG, Tiwari RP, Worsley PS, Dilworth MJ, Glenn AR, Howieson JG. 1999. Constructs for insertional mutagenesis , transcriptional signal localization and gene regulation studies in root nodule and other bacteria. *J. Bacteriol.*:1307–1316.

Rehm BHA, Mitsky TA. 2001. Role of Fatty Acid De Novo Biosynthesis in Polyhydroxyalkanoic Acid (PHA) and Rhamnolipid Synthesis by Pseudomonads : Establishment of the Transacylase (PhaG) -Mediated Pathway for PHA Biosynthesis in Escherichia coli. *Appl. Environ. Microbiol.* **67**:3102–3109.

Rhodius V a, Mutalik VK. 2010. Predicting strength and function for promoters of the Escherichia coli alternative sigma factor, sigmaE. *Proc. Natl. Acad. Sci. U. S. A.* **107**:2854–9.

Russell WM, Klaenhammer T. 2001. Identification and Cloning of gusA, Encoding a New β-Glucuronidase from Lactobacillus gasseri ADH. *Appl. Environ. Microbiol.* **67**:1253–1261.

Saborido Basconcillo L, Zaheer R, Finan TM, McCarry BE. 2009. Cyclopropane fatty acyl synthase in Sinorhizobium meliloti. *Microbiology* **155**:373–385.

Saruul P, Srienc F, Somers DA, Samac DA. 2002. Production of a Biodegradable Plastic Polymer, Poly-β-Hydroxybutyrate, in Transgenic Alfalfa. *Crop Sci.* **42**:919–927.

Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19:

selection of defined deletions in the chromosome of Corynebacterium glutamicum. *Gene* **145**:69–73.

Schallmey M, Ly A, Wang C, Meglei G, Voget S, Streit WR, Driscoll BT, Charles TC. 2011. genes from a soil metagenome library using phenotypic screening. *FEMS Microbiol. Lett.* **321**:150–156.

Schreck KM, Hillmyer MA. 2007. Block copolymers and melt blends of polylactide with Nodax TM microbial polyesters : Preparation and mechanical properties. *J. Biotechnol.* **132**:287–295.

Schubert P, Steinbuchel A, Schlegel HG. 1988. Cloning of the Alcaligenes eutrophus Genes for Synthesis of Poly-3-Hydroxybutyric Acid (PHB) and Synthesis of PHB in Escherichia coli. *J. Bacteriol.* **170**:5837–5847.

Selmer T, Willanzheimer A, Hetzel M. 2002. Propionate CoA-transferase from Clostridium propionicum Cloning of the gene and identification of glutamate 324 at the active site. *Eur. J. Biochem.* **380**:372–380.

Sendil D, Gursel I, Wise DL. 1999. Antibiotic release from biodegradable PHBV microparticles. *J. Control. Release* **59**:207–217.

Shozui F, Matsumoto K, Motohashi R, Sun J, Satoh T, Kakuchi T, Taguchi S. 2011. Biosynthesis of a lactate (LA)-based polyester with a 96mol% LA fraction and its application to stereocomplex formation. *Polym. Degrad. Stab.* **96**:499–504.

Shozui F, Matsumoto K, Motohashi R, Yamada M, Taguchi S. 2010a. Establishment of a metabolic pathway to introduce the 3-hydroxyhexanoate unit into LA-based polyesters via a reverse reaction of β-oxidation in Escherichia coli LS5218. *Polym. Degrad. Stab.* **95**:1340–1344.

Shozui F, Matsumoto K, Nakai T, Yamada M, Taguchi S. 2010b. Biosynthesis of novel terpolymers poly(lactate-co-3-hydroxybutyrate-co-3-hydroxyvalerate)s in lactate-overproducing mutant Escherichia coli JW0885 by feeding propionate as a precursor of 3-hydroxyvalerate. *Appl. Microbiol. Biotechnol.* **85**:949–954.

Slater S, Gallaher T, Dennis D. 1992. Production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) in a recombinant Escherichia coli strain. *Appl. Environ. Microbiol.* **58**:1089–1094.

Slater SC, Voige WH, Dennis DE, Biology D. 1988. Cloning and Expression in Escherichia coli of the Alcaligenes eutrophus H16 Poly-3-Hydroxybutyrate Biosynthetic Pathway. *J. Bacteriol.* **170**:4431–4436.

Slater S, Mitsky TA, Houmiel KL, Hao M, Reiser SE, Taylor NB, Tran M, Valentin HE, Rodriguez DJ, Stone DA, Padgette SR, Kishore G, Gruys KJ. 1999. Metabolic engineering

of Arabidopsis and Brassica for poly (3 -hydroxybutyrate- co -3- hydroxyvalerate) copolymer production. *Nat. Biotechnol.* **17**:1011–1016.

Snell KD, Feng F, Zhong L, Martin D, Madison LL. 2002. YfcX Enables Medium-Chain-Length Poly (3-Hydroxyalkanoate) Formation from Fatty Acids in Recombinant Escherichia coli fadB Strains. *J. Bacteriol.*:5696–5705.

Södergård A, Stolt M. 2002. Properties of lactic acid based polymers and their correlation with composition. *Prog. Polym. Sci.* **27**:1123–1163.

Spaink HP, Okker RJH, Wijffelman CA, Pees E, Lugtenberg BJJ. 1987. Promoters in the nodulation region of the Rhizobium leguminosarum Sym plasmid pRLIJI Herman. *Plant Mol. Biol.* **39**:27–39.

Stark MJR. 1987. Multicopy expression vectors carrying the fuc repressor gene for regulated high-level expression of genes in Escherichia coli (Recombinant DNA ; tat promoter ; IacI gene ; lac operator ; lacZa ; pUC plasmids ; polylinker ; gene fusion ; luciferase) A se. *Gene* **51**:255–267.

Sudesh K, Abe H, Doi Y. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**:1503–1555.

Sun Z, Ramsay J a, Guay M, Ramsay B a. 2007. Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by Pseudomonas putida KT2440. *Appl. Microbiol. Biotechnol.* **74**:69–77.

Taguchi K, Aoyagi Y, Matsusaki H, Fukui T, Doi Y. 1999. Over-expression of 3-ketoacyl-ACP synthase III or malonyl-CoA-ACP transacylase gene induces monomer supply for polyhydroxybutyrate production in Escherichia coli HB101. *Biotechnol. Lett.*:579–584.

Taguchi K, Tsuge T, Matsumoto K, Nakae S. 2001. Investigation of metabolic pathways for biopolyester production. *RIKEN Rev.* **42**:71–74.

Taguchi S, Yamada M, Tajima K, Satoh Y, Munekata M. 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **105**:1–5.

Tajima K, Satoh Y, Nakazawa K, Tannai H, Erata T, Munekata M, Kamachi M, Lenz RW. 2004. Chemoenzymatic Synthesis of Poly(3-hydroxybutyrate) in a Water-Organic Solvent Two-Phase System. *Macromolecules* **37**:4544–4546.

Timm A, Steinbuchel A. 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of Pseudomonas aeruginosa PAO1. *Eur. J. Biochem.* **209**:15–30.

Timm A, Wiese S, Steinbiichei A. 1994. A general method for identification of polyhydroxyalkanoic acid synthase genes from pseudomonads belonging to the rRNA homology group I. *Appl. Microbiol. Biotechnol.* **1**:669–675.

Timmis KN. 2002. Pseudomonas putida : a cosmopolitan opportunist par excellence. *Environ. Microbiol.* **4**:779–781.

Trainer MA, Capstick D, Zachertowska A, Lam KN, Clark SRD, Charles TC. 2010. Identification and characterization of the intracellular poly-3-hydroxybutyrate depolymerase enzyme PhaZ of Sinorhizobium meliloti. *BMC Microbiol.* **10**:1–10.

Trainer MA, Charles TC. 2006. The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Appl. Microbiol. Biotechnol.* **71**:377–386.

Tsuge T, Fukui T, Matsusaki H, Taguchi S, Kobayashi G, Ishizaki A, Doi Y. 2000. Molecular cloning of two (R)-specific enoyl-CoA hydratase genes from Pseudomonas aeruginosa and their use for polyhydroxyalkanoate synthesis. *FEMS Microbiol. Lett.* **184**:193–188.

Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y. 2004. Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-co-(3-hydroxyhexanoate)] in recombinant ralstonia eutropha expressing mutated polyhydroxyalkanoate synthase genes. *Macromol. Biosci.* **4**:238–242.

Valentin HE, Broyles DL, Casagrande LA, Colburn SM, Creely WL, DeLaquil PA, Felton HM, Gonzalez K a., Houmiel KL, Lutke K, Mahadeo DA, Mitsky TA, Padgette SR, Reiser SE, Slater S, Stark DM, Stock RT, Stone DA, Taylor NB, Thorne GM, Tran M, Gruys KJ. 1999. PHA production, from bacteria to plants. *Int. J. Biol. Macromol.* **25**:303–306.

Wang Y-W, Wu Q, Chen J, Chen G-Q. 2005. Evaluation of three-dimensional scaffolds made of blends of hydroxyapatite and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) for bone reconstruction. *Biomaterials* **26**:899–904.

Wong MS, Causey TB, Mantzaris N, Bennett GN, San K. 2008. Engineering Poly (3-hydroxybutyrate- co -3- hydroxyvalerate) Copolymer Composition in E . coli. *Biotechnol. Bioeng.* **99**:919–928.

Yagmurlu MF, Korkusuz F, Gu I, Korkusuz P, Örs Ü, Hasirci V. 1999. hydroxyvalerate (PHBV) local antibiotic delivery system : In vivo effectiveness and biocompatibility in the treatment of implant-related experimental osteomyelitis. *J. Biomed. Mater. Res.* **46**:494–503.

Yamada M, Matsumoto K, Shimizu K, Uramoto S, Nakai T, Shozui F, Taguchi S. 2010. Adjustable Mutations in Lactate (LA) -Polymerizing Enzyme for the Microbial Production of LA-Based Polyesters with Tailor-Made Monomer Composition. *Biomacromolecules*:815–819.

Yang TH, Jung YK, Kang HO, Kim TW, Park SJ, Lee SY. 2011. Tailor-made type II Pseudomonas PHA synthases and their use for the biosynthesis of polylactic acid and its copolymer in recombinant Escherichia coli. *Appl. Microbiol.*

Yang TH, Kim TW, Kang HO, Lee S-H, Lee EJ, Lim S-C, Oh SO, Song A-J, Park SJ, Lee SY. 2010. Biosynthesis of polylactic acid and its copolymers using evolved propionate CoA transferase and PHA synthase. *Biotechnol. Bioeng.* **105**:150–160.

Yim KS, Lee SY, Chang HN. 1996. Synthesis of Poly- (3-Hydroxybutyrate-co- 3-Hydroxyvalerate) by Recombinant Escherichia coli. *Biotechnol. Bioeng.* **49**:495–503.

Yunus AMM, Ghulam KAP, Ho C. 2008. Transgenic Plants Producing Polyhydroxyalkanoates. *Asia Pacific J. Mol. Biol. Biotechnol.* **16**:1–10.

Zhang B, Carlson R, Srienc F. 2006. Engineering the monomer composition of polyhydroxyalkanoates synthesized in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **72**:536–543.

Zhang X, Luo R, Wang Z, Deng Y, Chen G-Q. 2009. Application of (R)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. *Biomacromolecules* **10**:707–711.

Zhao K, Deng Y, Chun Chen J, Chen G-Q. 2003. Polyhydroxyalkanoate (PHA) scaffolds with good mechanical properties and biocompatibility. *Biomaterials* **24**:1041–1045.