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Review Article

PRODUCTION OF PHA BY RECOMBINANT ORGANISMS

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Polyhydroxyalkanoates (PHA) are biodegradable, thermoplastic polyesters produced from renewable carbon sources by a number of bacteria. However, their application is limited by high production costs. One of the strategies aimed to reduce their costs is the development of recombinant strains able to utilize different carbon sources. Many bacteria are naturally capable of accumulating biopolyesters composed of 3-hydroxy fatty acids as intracellular inclusions, which serve as storage granules. Recently, these inclusions have been considered as nano/microbeads with surface-attached proteins, which can be engineered to display various protein-based functions that are suitable for biotechnological and biomedical applications. Natural PHA producers have become accustomed to accumulating PHA during evolution; they often have a long generation time, relatively low optimal growth temperature, are often hard to lysis and contain pathways for PHA degradation. This led to the development of recombinant PHA producers, capable of high PHA accumulation and/or free of PHA degradative pathways. This review summarizes the production of PHA by recombinant bacteria as well as some higher organisms in brief.

Keywords: Polyhydroxyalkanoates, Production, Recombinant bacteria

INTRODUCTION

Natural PHA producers have become accustomed to accumulating PHA during evolution; they often have a long generation time, relatively low optimal growth temperature, are often hard to lysis and contain pathways for PHA degradation. This led to the development of recombinant PHA producers, capable of high PHA accumulation and/or free of PHA degradative pathways. Polyhydroxyalkanoates (PHAs) are biodegradable, thermoplastic polyesters produced from renewable carbon sources by a number of bacteria. However, their application is limited by high production costs. One of the strategies aimed to reduce their costs is the development of recombinant strains able to utilize different carbon sources.

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METABOLIC ENGINEERING OF PHA BIOSYNTHESIS IN DIFFERENT BACTERIA

Cupriavidus necator or *Alcaligenes eutrophus* (formerly *Ralstonia eutropha*)

Recombinant *Ralstonia eutropha* cells over expressing *phaCAB* genes from a plasmid, showed increase in P (3HB) levels from 33-40% of the cell dry weight (Park et al., 1995). Although the increase was insignificant, recombinant *R.eutropha* strains could reduce the fermentation time by 20% while maintaining the same productivity (Park *et al*., 1997). This is significant from commercial production point of view, since the overall productivity of a P (3HB) plant would be 20% higher.

Natural producers like *Cupriavidus necator* produces high levels of P (3HB), but they have certain limitations. *Cupriavidus necator grows* slowly, it is difficult to lyse and is genetically not well characterized which impedes genetic manipulations. As against this, *Escherichia coli* is genetically well characterized. Not being a natural PHA accumulator, PHA production has to be metabolically engineered in *E. coli* and it does not have any depolymerase activity to degrade accumulated PHA.

Synthesis of P(HB-*co*-HHx) from plant oil has been demonstrated with recombinant *Ralstonia eutropha* strains expressing heterologous PHA synthases capable of incorporating HB and HHx into the polymer. With these strains, however, short-chain-length fatty acids had to be included in the medium to generate PHA with high HHx content. Two *R. eutropha* strains have been engineered that accumulate high levels of P(HB-*co*-HHx) with significant HHx content directly from palm oil, one of the world's most abundant plant oils. The strains express a newly characterized PHA synthase gene from the bacterium *Rhodococcus aetherivorans* I24.

Cupriavidus necator DSM 545 is a well-known polyhydroxyalkanoates (PHAs) producer, but unable to grow on lactose. The aim of this study was to construct a recombinant strain of *C. necator* that can use lactose-containing waste material such as cheese whey, to produce PHAs. One of the intracellular PHA depolymerases (*phaZ1*) of *C. necator* was chosen to insert the *lacZ*, *lacl* and *lacO* genes of *Escherichia coli*. This would have the effect to allow polymer production on lactose and, at the same time, to remove part of the PHA intracellular degradation system. Disruption of *phaZ1* was achieved by gene replacement after isolating a fragment of this gene and interrupting it with a cartridge containing the *lac* genes and a synthetic promoter. Growth and polymer production studies of the genetically modified (GM) strain mRePT in lactose, whey permeate and hydrolyzed whey permeate as carbon sources, were performed. Lower PHA degradation and higher yields were obtained compared to the wild-type strain. Inactivation of the putative depolymerase gene *phaZ3* on mRePT recombinant strain was also reported (Silvana Povolo *et al*., 2010).

Expression of an enoyl coenzyme A (enoyl-CoA) hydratase gene (*phaJ*) from *Pseudomonas aeruginosa* was shown to increase PHA accumulation. Furthermore, varying the activity of acetoacetyl-CoA reductase (encoded by *phaB*) altered the level of HHx in the polymer. The strains with the highest PHA titers utilized plasmids for recombinant gene expression, so an *R. eutropha* plasmid stability system was developed. In this system, the essential pyrroline-5-carboxylate reductase gene *proC* was deleted from strain genomes and expressed from a plasmid, making

the plasmid necessary for growth in minimal media. This study resulted in two engineered strains for production of P(HB-*co*-HHx) from palm oil. In palm oil fermentations, one strain accumulated 71% of its cell dry weight as PHA with 17 mol% HHx, while the other strain accumulated 66% of its cell dry weight as PHA with 30 mol% HHx (Charles *et al.*, 2011).

Cupriavidus necator was engineered aiming to synthesize poly[(*R*)-3-hydroxybutyrate-*co*-3-hydroxypropionate] copolyester, P(3HB-*co*-3HP), from structurally unrelated carbon sources without addition of any precursor compounds. We modified a metabolic pathway in *C. necator* for generation of 3-hydroxypropionyl-CoA (3HP-CoA) by introducing malonyl-CoA reductase and the 3HP-CoA synthetase domain of trifunctional propionyl-CoA synthase; both members of the 3-hydroxypropionate cycle, a novel CO_2-fixation pathway in the green nonsulfur bacterium *Chloroflexus aurantiacus*. In this recombinant strain, 3HP-CoA was expected to be provided from acetyl-CoA via malonyl-CoA, and then copolymerized by the function of polyhydroxy-alkanoate synthase along with (*R*)-3-hydroxybutyryl-CoA synthesized from two acetyl-CoA molecules. *C. necator* wild-type strains H16 and JMP134 harboring the two heterologous genes actually synthesized P(3HB-*co*-3HP) copolyester with 0.2-2.1 mol % of 3HP fraction from fructose or alkanoic acids of even carbon numbers. Enzyme assay suggested that lower activity of 3HP-CoA synthetase than that of malonyl-CoA reductase caused the limited incorporation of 3HP unit into the copolyesters synthesized by the recombinant strains. The present study demonstrates the potential of engineering metabolic pathways for production of copolyesters having favorable characteristics from inexpensive carbon resources (Toshiaki Fukui *et al.*, 2009).

Unlike polyhydroxyalkanoates (PHAs) copolymers, the controlled and efficient synthesis of PHA terpolymers from triglycerides and fatty acids are yet to be established. This study demonstrates the production of P(3HB-*co*-3HV-*co*-3HHx) terpolymer with a wide range of 3HV monomer compositions from mixtures of crude palm kernel oil and 3HV precursors using a mutant *Cupriavidus necator* PHB-4 transformant harboring the PHA synthase gene (*phaC*) of a locally isolated *Chromobacterium* sp. USM2. The PHA synthase of *Chromobacterium* has an unusually high affinity towards 3HV monomer. P(3HB-*co*-3HV-*co*-3HHx) terpolymers with 3HV monomer composition ranging from 2 to 91 mol% were produced. Generation of 3HHx monomers was affected by the concentration and feeding time of 3HV precursor. P(3HB-*co*-24 mol% 3HV-*co*-7 mol% 3HHx) exhibited mechanical properties similar to that of common low-density polyethylene. P(3HB-*co*-3HV-*co*-3HHx) terpolymers with a wide range of 3HV molar fraction had been successfully synthesized by adding lower concentrations of 3HV precursors and using a PHA synthase with high affinity towards 3HV monomer (Kesaven Bhubalan *et al.*, 2010).

Escherichia coli

Polyhydroxyalkanoates (PHAs) are biodegradable plastics. Currently most PHAs are produced using microbial fermentation. The separation of PHAs is a key factor in the design of a successful production scheme. This study chose a recombinant *Escherichia coli* to produce poly(3-hydroxybutyrate) (PHB), an important one among PHAs; and to use different separation methods to recover PHB. The diversity of PHB obtained by different separation methods was characterized. Among different methods used, sodium dodecyl sulfate (SDS)–NaOH method

was selected as the best in separation from the results obtained from experiments using a combinatorial design. In the combinatorial design, only 16 runs are needed to generate 150 sets of experimental data. By using a two-stage design, PHB with a purity of 99% was obtained when the first-stage digestion used SDS digestion method (50 °C, 125 rpm, biomass/digestion solution (100 g/L) = 1/10 (w/v) and 10 min) and the second-stage used NaOH method (50 °C, 125 rpm, biomass/digestion solution (1 M) = 1/10 (w/v) and 10 min) (Chi-Wei Lo *et al.*, 2011).

To develop an approach to enhance polyhydroxybutyrate (PHB) production *via* the coexpressed *phaCAB* and *vgb* genes controlled by arabinose P_{BAD} promoter in *Escherichia coli*. The polyhydroxyalkanoates (PHAs) synthesis operon, (*phaCAB*), from *Ralstonia eutropha* was overexpressed under the regulation of the arabinose P_{BAD} promoter in *Escherichia coli,* and the *vgb* gene encoding bacterial haemoglobin from *Vitreoscilla stercoraria* (VHb) was further cloned at downstream of *phaCAB* to form an artificial operon. The cell dry weight (CDW), PHB content and PHB concentration were enhanced around 1·23-, 1·57-, and 1·93-fold in the engineered cell harbouring *phaCAB–vgb* (SY-2) upon 1% arabinose induction compared with noninduction (0% arabinose). Furthermore, by using a recombinant strain harbouring P_{BAD} promoter-*vgb* along with native promoter-*phaCAB* construction, the effect of *vgb* expression level on PHB biosynthesis was positive correlation (Horng *et al.*, 2010).

The synthesis of bacterial polyhydroxy-alkanoates (PHA) is very much dependent on the expression and activity of a key enzyme, PHA synthase (*PhaC*). Many efforts are being pursued to enhance the activity and broaden the substrate specificity of *PhaC*. Here, the identification of a highly active wild-type *PhaC* belonging to the recently isolated *Chromobacterium* sp. USM2 (PhaC$_{Cs}$) was reported. PhaC$_{Cs}$ showed the ability to utilize 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) monomers in PHA biosynthesis. An *in vitro* assay of recombinant PhaC$_{Cs}$ expressed in *Escherichia coli* showed that its polymerization of 3-hydroxybutyryl-coenzyme A activity was nearly 8-fold higher (2,462 ± 80 U/g) than that of the synthase from the model strain *C. necator* (307 ± 24 U/g). Specific activity using a Strep2-tagged, purified PhaC$_{Cs}$ was 238 ± 98 U/mg, almost 5-fold higher than findings of previous studies using purified PhaC from *C. necator*. Efficient poly(3-hydroxybutyrate) [P(3HB)] accumulation in *Escherichia coli* expressing PhaC$_{Cs}$ of up to 76 ± 2 weight percent was observed within 24 h of cultivation. To date, this is the highest activity reported for a purified PHA synthase. PhaC$_{Cs}$ is a naturally occurring, highly active PHA synthase with superior polymerizing ability (Kesaven Bhubalan *et al.*, 2011).

The strategic design of this study aimed at producing succinate and polyhydroxyalkanoate (PHA) from substrate mixture of glycerol/glucose and fatty acid in *Escherichia coli*. To accomplish this, an *E. coli* KNSP1 strain derived from *E. coli* LR1110 was constructed by deletions of *ptsG*, *sdhA* and *pta* genes and overexpression of *phaC*1 from *Pseudomonas aeruginosa*. Cultivation of *E. coli* KNSP1 showed that this strain was able to produce 21.07 g/L succinate and 0.54 g/L PHA (5.62 wt.% of cell dry weight) from glycerol and fatty acid mixture. The generated PHA composed of 58.7 mol% 3-hydroxyoctanoate (3HO) and 41.3 mol% 3-hydroxydecanoate (3HD). This strain would be useful for complete utilization of byproducts glycerol and fatty acid of biodiesel production process (Zhen Kang et al., 2011).

The industrial production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) has been hindered by high cost and a complex control strategy caused by the addition of propionate. In this study, based on analysis of the PHBV biosynthesis process, PHBV biosynthetic pathway was developed from a single unrelated carbon source via threonine biosynthesis in *Escherichia coli*. To accomplish this, (i) overexpression of threonine deaminase was done, which is the key factor for providing propionyl-coenzyme A (propionyl-CoA), from different host bacteria, (ii) removal of the feedback inhibition of threonine was done by mutating and overexpressing the *thrABC* operon in *E. coli*, and (iii) knocking out the competitive pathways of catalytic conversion of propionyl-CoA to 3-hydroxyvaleryl-CoA was done. Finally, construction of a series of strains and mutants which were able to produce the PHBV copolymer with differing monomer compositions in a modified M9 medium supplemented with 20 g/liter xylose was done. The largest 3-hydroxyvalerate fraction obtained in the copolymer was 17.5 mol% (Quan Chen *et al.*, 2011).

The optimal conditions for 3-hydroxybutyrate (P[3HB]) production by the recombinant *Escherichia coli* strain JM101, harboring *Ralstonia eutropha* PHA biosynthesis genes, in a defined medium containing hydrolyzed corn starch and soybean oil as carbon sources and cheese whey as a supplement had been established earlier. In order to optimize PHA production, the present study investigated the influence of other variables, such as isopropyl-1-thio-b-D-galactopyranoside (IPTG) and acrylic acid addition, temperature, inoculum size and cultivation time, on dry cell weight (DCW) and PHA accumulation through a super factorial design. Statistical models suggested that quite good PHA production performances would be obtained in *E. coli* JM101 cultures grown in a medium lacking IPTG and acrylic acid with an inoculum size of 5% (v/v) and performed at 37C for 96 h. Theoretically, about 3.5 g L super(–1) DCW and 75% PHA could be accumulated. Acrylic acid had a negative effect on DCW at its high level (1 mmol) and was unable to promote incorporation of medium-chain-length units into PHA polymers as expected. Remarkably, IPTG, a very expensive inducer of PHA synthesis gene expression had no influence on PHA production (Fonseca and Antonio, 2010).

Expression of *Pseudomonas aeruginosa* PHA synthases, *phaC1* and *phaC2* in *E.coli fadB* mutant resulted in msc-PHA accumulation when grown in presence of C8-C14 fatty acids. The *E. coli fadB* mutant LS1298 containing *phaC1Pa* gene accumulated 21% PHA of cellular dry weight composed of mainly 3-hydroxydecanoate (72.5 mol%) and 3-hydroxyoctanoate (20 mol%) when grown in LB medium containing decanoate. Using acrylic acid, intermediates of fatty acid synthesis were chanelled to PHA synthesis in recombinant E. coli harboring the *phaC1Pa* gene (Qi et al., 1998).

Escherichia coli CGSC 4401 harboring pJC4, a plasmid containing the *Alcaligenes latus* PHA biosynthesis genes when grown on whey by fed-batch culture accumulated PHB. With lactose below 2g/l, cells grew to 12g dry cell weight/l with 9% (w/w) P(3HB) content, increasing lactose to 20g/l increased PHB content to 70% after 26 h growth (Park et al., 2002). The author's earlier work with the same recombinant strain grown on concentrated whey containing 280 g of lactose equivalent per litre produced 119.5 and 96.2g/l, final cell and PHB concentrations with PHB productivities of 2.57g/l/h (Park et al., 2002).

PhaC from *Wautersia eutropha* expressed in a wide range of production level in *Escherichia coli* XL1-Blue and its effects on *PhaC* activity, PHB production and its molecular weights were reported by Agus *et al.* (2006a). The results revealed that the production level of the biosynthesis genes was controlled both by the amount of chemical inducer (isopropyl-β-D-thiogalactopyranoside, IPTG) added into the medium and the use of different copy number of plasmids. The molecular weight of P (3HB) was found to decrease with increasing *PhaC* activity.

Bioreactor cultures of *Escherichia coli* recombinants carrying *phaBAC* and *phaP* of *Azotobacter* sp. FA8 grown on glycerol under low-agitation conditions accumulated more poly(3-hydroxybutyrate) (PHB) and ethanol than at high agitation, while in glucose cultures, low agitation led to a decrease in PHB formation. Cells produced smaller amounts of acids from glycerol than from glucose. Glycerol batch cultures stirred at 125 rpm accumulated, in 24 h, 30.1% (wt/wt) PHB with a relative molecular mass of 1.9 MDa, close to that of PHB obtained using glucose (Alejandra de Almeida et al., 2010).

NAD kinase was over expressed to enhance the accumulation of poly(3-hydroxybutyrate) (PHB) in recombinant *Escherichia coli* harboring PHB synthesis pathway via an accelerated supply of NADPH, which is one of the most crucial factors influencing PHB production. A high copy number expression plasmid pE76 led to a stronger NAD kinase activity than that brought about by the low copy number plasmid pELRY. Overexpressing NAD kinase in recombinant *E. coli* was found not to have a negative effect on cell growth in the absence of PHB synthesis. Shake flask experiments demonstrated that excess NAD kinase in *E. coli* harboring the PHB synthesis operon could increase the

accumulation of PHB to 16-35 wt. % compared with the controls; meanwhile, NADP concentration was enhanced threefold to sixfold. Although the two NAD kinase overexpression recombinants exhibited large disparity on NAD kinase activity, their influence on cell growth and PHB accumulation was not proportional. Under the same growth conditions without process optimization, the NAD kinase-overexpressing recombinant produced 14 g/L PHB compared with 7 g/L produced by the control in a 28-h fermentor study. In addition, substrate to PHB yield $Y_{PHB/glucose}$ showed an increase from 0.08 g PHB/g glucose for the control to 0.15 g PHB/g glucose for the NAD kinase-overexpressing strain, a 76% increase for the $Y_{PHB/glucose}$. These results clearly showed that the overexpression of NAD kinase could be used to enhance the PHB synthesis (Zheng-Jun Li).

We have developed the conversion of glycerol into thermoplastic poly(3-hydroxypropionate) [poly(3HP)] was developed. For this, the genes for glycerol dehydratase (*dhaB1*) of *Clostridium butyricum*, propionaldehyde dehydrogenase (*pduP*) of *Salmonella enterica* serovar Typhimurium LT2, and polyhydroxyalkanoate (PHA) synthase (*phaC1*) of *Ralstonia eutropha* were expressed in recombinant *Escherichia coli*. Poly(3HP) was accumulated up to 11.98% (wt/wt [cell dry weight]) in a two-step, fed-batch fermentation. The present study shows an interesting application to engineer a poly(3HP) synthesis pathway in bacteria (Björn Andreeßen et al., 2010).

Based on the metabolic analysis, a succinate and polyhydroxybutyrate (PHB) co-production pathway was designed and engineered in *Escherichia coli* MG1655. Batch cultivation of the engineered *E. coli* revealed that it was able to accumulate both extracellular succinate and

intracellular PHB simultaneously. PHB accumulation not only improved succinate production, but also reduced pyruvate and acetate secretion. With a consumption of 45 g l^{-1} glucose, *E. coli* QZ1112 was shown to accumulate 24.6 g l^{-1} succinate and 4.95 g l^{-1} PHB in batch fermentation. The PHB content reached 41.3 wt.% of its cell dry weight, which suggested that the cell debris can be used as value added by-product (Zhen Kang et al., 2010).

The pH-stat fed-batch culture of XL1-Blue achieved a PHB concentration of 88.8 g/l in 42 h.Similarly, a PHB concentration of 81.2 g/l was obtained in 39h by fed-batch culture of XL1-Blue harboring a stable high-copy-number plasmid. However, fed-batch culture of XL1-Blue harboring a stable medium-copy-number plasmid resulted in only 30.5 g/l of PHB in 41 h, suggesting that a high gene dosage obtained by using high-copy-number plasmid is required for the higher accumulation of PHB in recombinant *E. coli* (Lee and Chang, 1995). Lee and Chang (1995) transformed a number of *E coli* strains with a stable high-copy-number plasmid containing the *R. eutropha* PHB biosynthesis genes, and compared these for their ability to synthesize and accumulate PHB.

E. coli JM109 accumulated 85% PHB of cell dry weight, while XL1-Blue accumulated 81% PHB of cell dry weight. However, yield per gram of glucose was higher for XL1-Blue (0.37 g PHB/g glucose) than for JM109 (0.3 g PHB/g glucose). Cultivation of recombinant *E. coli* in defined medium led to reduced levels of PHB accumulation. Supplementation of complex nitrogen sources to defined media restores the normal levels of PHB accumulation (Lee and Chang, 1995). For cost reduction purpose, cheap carbon sources such as molasses, whey etc. have been used for PHA production. For

enhanced PHA production in defined media by recombinant *E. coli*, *ftsZ* gene was expressed in *E .coli* to suppress the filamentation (Lee and Lee, 1996).

Expression of *R. eutropha* PHA synthase gene and the *Clostridium kluyveri orfZ* encoding for 4-hydroxybutyrate-CoA transferase in *E. coli* led to the accumulation of P(4HB) homopolymer, when grown in presence of glucose. In the absence of glucose, a P(3HB-4HB) copolymer accumulated with up to 72% 3HB incorporation, even though *phbA* and *phaB* were absent. This indicates that in *E. coli* an unknown pathway allows the conversion of 4HB to 3HB (Hein et al., 1997). Valentin and Dennis (Valentin and Dennis, 1997) co-expressed succinate degradation pathway from *Clostridium kluyveri* and PHB biosynthetic pathway from *R. eutropha* in *E. coli* resulting recombinant accumulated 46% P (3HB-4HB) of cell dry weight with 1.5% 4HB incorporation. To provide 4(HB)-CoA for poly (3HB-4HB) synthesis from glutamate, an acetyl-CoA:4-hydroxybutyrate CoA transferase from *C. kluyveri*, a 4-hydroxybutyrate dehydrogenase from *R. eutropha*, a α-aminobutyrate: 2-ketoglutarate transaminase from *Escherichia coli* and glutamate decarboxylases from *Arabidopsis thaliana* or *E. coli* were expressed in succinic semialdehyde mutant *E. coli* CT 101 (Valentin et al., 2000).

Nine anaerobic promoters were cloned and constructed upstream of PHB synthesis genes *phbCAB* from *Ralstonia eutropha* for the micro- or anaerobic PHB production in recombinant *Escherichia coli*. Among the promoters, the one for alcohol dehydrogenase (P$_{adhE}$) was found most effective. Recombinant *E. coli* JM 109 (pWCY09) harboring P$_{adhE}$ and *phbCAB* achieved a 48% PHB accumulation in the cell dry weight after 48 h of static culture compared with only

30% PHB production under its native promoter. Sixty-seven percent PHB was produced in the dry weight (CDW) of an acetate pathway deleted (Δpta deletion) *E. coli* JW2294 harboring the vector pWCY09. In a batch process conducted in a 5.5-l NBS fermentor containing 3 l glucose LB medium, *E. coli* JW2294 (pWCY09) grew to 7.8 g/l CDW containing 64% PHB after 24 h of microaerobic incubation. In addition, molecular weight of PHB was observed to be much higher under microaerobic culture conditions. The high activity of P_{adhE} appeared to be the reason for improved micro- or anaerobic cell growth and PHB production while high molecular weight contributed to the static culture condition (Xiao-Xing Wei et al.).

A variety of polymers, such as, P(3HB-3HV), P(3HB-4HB), P(4HB) and P(3HO-3HH) have been synthesized by genetically and metabolically engineered *E. coli*. During PHBV production, incorporation of 3HV by recombinant *E.coli* requires the function of *ackA* (acetate kinase) and *pta* (phosphotransacetylase) genes (Rhie and Dennis, 1995). The expression of PHA biosynthetic genes of *Cupriavidus necator* in *E. coli* for P (3HB) synthesis opened up the avenues for PHA production by recombinant organisms (Slater et al., 1998, Peoples and Sinskey, 1989b). Propionate was provided for the PHBV production in recombinant E. *coli*. Since *E. coli* does not easily import propionate, cultures were adapted on acetate and then a glucose-propionic acid mixture was added. Further, this system was improved by using E. *coli* strains that have constitutive expression of the *ato* operon and *fad* regulon to fully express fatty acid utilization enzymes (Slater et al., 1992; and Fidler and Dennis, 1992). The 3HV fraction in the copolymer was dependent on the percentage of propionate used during the fermentation. *E. coli* is resistant to 100 mM propionate, while, 30 mM is already toxic to *R. eutropha* (Ramsay et al., 1990), making PHBV fermentations more efficient with *E .coli* strains.

Recombinant *E. coli* cells harboring *A. latus* PHA biosynthetic genes were grown in fed-batch culture with the pH-stat feeding strategy in a chemically defined medium (Choi and Lee, 1999b). An improved nutrient feeding strategy to obtain glucose and propionic acid concentration at 110 mM and 20 mM respectively, acetic acid induction and oleic acid supplementation led to high level of PHBV production, 2.88 g of P(3HB-3HV)/l/h. Valladares *et al.* (2006) demonstrated that it is *RHlAB* (a gene for rhamnolipid biosynthesis) specificity and not the hydroxy fatty acid relative abundance in the bacterium, that determines the profile of the fatty acid moiety of rhamnolipids and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAA) in recombinant *E.coli* strain expressing *Pseudomonas aeruginosa rhlAB* operon. Production of R-3-hydroxybutyric acid (3HB) was observed when genes of α-ketothiolase (*PhbA*), acetoacety-CoA reductase (*PhbB*) and thioesterase II (*TesB*) when jointly expressed in *E.coli* produced approximately 4 g/l 3HB in shake flask culture within 24 hours with glucose as a carbon source. Under anaerobic conditions PHB production increased to 0.47 g 3HB/g glucose. Sodium gluconate yielded 6 g/l.

This study evaluates the effect of *PhaP*, a PHA gene, on bacterial growth and PHB accumulation from glycerol in bioreactor cultures of recombinant *Escherichia coli* carrying *phaBAC* from *Azotobacter* sp. strain FA8 was studied. Cells expressing *phaP* grew more, and accumulated more PHB, both using glucose and using glycerol as carbon sources. When cultures were grown in a bioreactor using glycerol, *PhaP*-bearing cells produced more polymer (2.6 times) and more biomass (1.9 times) than did those without it. The effect of this protein on growth promotion and polymer accumulation is expected

to be even greater in high-density cultures, such as, those used in the industrial production of the polymer. The recombinant strain presented in this work has been successfully used for the production of PHB from glycerol in bioreactor studies, allowing the production of 7.9 g/liter of the polymer in a semisynthetic medium in 48-h batch cultures (Alejandra et al., 2007).

Pseudomonas

Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by a wide range of bacteria, including Pseudomonads. These polymers are accumulated in the cytoplasm as carbon and energy storage materials when culture conditions are unbalanced and hence, they have been classically considered to act as sinks for carbon and reducing equivalents when nutrients are limited. Bacteria facing carbon excess and nutrient limitation store the extra carbon as PHAs through the PHA polymerase (PhaC). Thereafter, under starvation conditions, PHA depolymerase (PhaZ) degrades PHA and releases *R*-hydroxyalkanoic acids, which can be used as carbon and energy sources. To study the influence of a deficient PHA metabolism in the growth of *Pseudomonas putida* KT2442 we have constructed two mutant strains defective in PHA polymerase (*phaC1*)- and PHA depolymerase (*phaZ*)-coding genes respectively. By using these mutants we have demonstrated that PHAs play a fundamental role in balancing the stored carbon/biomass/number of cells as function of carbon availability, suggesting that PHA metabolism allows *P. putida* to adapt the carbon flux of hydroxyacyl-CoAs to cellular demand. Furthermore, we have established that the coordination of PHA synthesis and mobilization pathways configures a functional PHA turnover cycle in *P. putida* KT2442. Finally, a new strain

able to secrete enantiomerically pure *R*-hydroxyalkanoic acids to the culture medium during cell growth has been engineering by redirecting the PHA cycle to biopolymer hydrolysis (Laura Isabel De Eugenio et al., 2010).

In expensive substrates like tallow, low rank coal liquefaction products, palm kernel oil etc. (Cromwick et al., 1996; Tan IKP et al., 1997; Füchtenbusch and Steinbüchel , 1999) has been tested for PHA production by *Pseudomonas* sp. Continuous production of PHA by *P. oleovorans*, using octanoate as a carbon source in a one-stage culture was shown to produce 0.15 g/l/h (Ramsay et al., 1991). In continuous cultivation systems, *P.oleovorans* is reported to produce 0.58 g/l/h PHA (Preusting et al., 1993) while 1.56g/l/h of PHA yield is reported for *Pseudomonas putida* (Hazenberg and Witholt, 1997). *Pseudomonas stutzeri* 1317, isolated from oil contaminated soil was found to grow well in glucose and soya bean oil as a sole carbon source. PHA containing mcl monomers ranging from C6-C14 were synthesized upto 52% of cell dry weight when grown on glucose mineral media. Upto 63% PHA containing novel monomer of 3, 6-epoxy-7-nonane-1,9 dioic acids with minor monomers of C8 and C10 (He et al., 1998).

The operon comprising the genes for poly-α-hydroxybutyrate (PHB) biosynthesis in *Pseudomonas* sp BJ-1 was cloned and sequenced. Sequence analysis of 8991 bp revealed that the regions contain two related operons. The first operon contains the three genes *phbA*, *phbB* and *phbC*, and the other contains the two genes *flp1* and *flp2*. The deduced amino acid sequences of PHBA and PHBB showed high identity with other bacterial PHB genes. Transcription of the three genes of the first operon is controlled by a single hypothetical promoter

region, whereas the other two *flp* genes are controlled by two hypothetical pro-moter regions. Analysis of expressed protein at different times showed that PHBA protein levels increased from 0 to 4 h; PHBB and PHBC showed similar kinetics. Detection of enzyme activity showed three proteins with bioactivity and biological function in the synthesis of PHB intermediates (Zhu et al., 2010).

Polyhydroxyalkanoate (PHA) synthesis genes phaPCJAc cloned from *Aeromonas caviae* were transformed into *Pseudomonas putida* KTOY06ΔC, a mutant of *P.putida* KT2442, resulting in the ability of the recombinant *P. putida* KTOY06ΔC (phaPCJA.c) to produce a short-chainlength and medium-chain-length PHA block copolymer consisting of poly-3-hydroxybutyrate (PHB) as one block and random copolymer of 3-hydroxyvalerate (3HV) and 3-hydroxyheptanoate (3HHp) as another block. The novel block polymer was studied by differential scanning calorimetry (DSC), nuclear magnetic resonance, and rheology measurements. DSC studies showed the polymer to possess two glass transition temperatures (Tg), one melting temperature (Tm) and one cool crystallization temperature (Tc). Rheology studies clearly indicated a polymer chain re-arrangement in the copolymer; these studies confirmed the polymer to be a block copolymer, with over 70 mol% homopolymer (PHB) of 3-hydroxybutyrate (3HB) as one block and around 30 mol% random copolymers of 3HV and 3HHp as the second block. The block copolymer was shown to have the highest tensile strength and Young's modulus compared with a random copolymer with similar ratio and a blend of homopolymers PHB and PHVHHp with similar ratio. Compared with other commercially available

PHA including PHB, PHBV, PHBHHx, and P3HB4HB, the short-chain- and medium-chain-length block copolymer PHB-b-PHVHHp showed differences in terms of mechanical properties and should draw more attentions from the PHA research community (Shi Yan Li et al.).

Pseudomonas putida KT2442 produces medium-chain-length (MCL) polyhydroxy-alkanoates (PHA) from fatty acids. When gene encoding 3-hydroxyacyl-CoA dehydrogenase which catalyzes long-chain-3-hydroxyacyl-CoA to 3-ketoacyl-CoA, was partially or completely deleted in *P. putida* KTOY08, the PHA accumulated was shown to contain only two different monomer structures dominated by a monomer of the same chain length as that of the fatty acids fed and another monomer two carbon atoms shorter. Among the PHA copolymers, P(44% 3HD-*co*-3HDD) containing 44% 3HD and 56% 3HDD was demonstrated to have a melting temperature T_m, an apparent heat of fusion ΔH_m and a Young's modulus E of 75 °C, 51 J g^{-1} and 2.0 MPa, respectively, the highest among all the MCL PHA studied (Long Ma et al., 2009). *Pseudomonas putida* KT2440 growing on nonanoic acid under nitrogen limitation produced 27% mcl PHA at specific ratio of 0.48/h. In contrast, exponential nonanoic acid-limited growth (0.15/h) yielded 75% PHA. The authors concluded that, the nitrogen limitation was unnecessary for the production of mcl PHA by the bacterium under carbon limited fed-batch production (Sun et al., 2007). *fadBA* operon in the fattyacid β-oxidation pathway of *P. putida* KCTC 1639, when blocked, induced a metabolic flux of intermediates to the biosynthesis of medium chain length PHA. Succinate at 150 mg/l stimulated cell growth and also biosynthesis of mcl PHA which yielded 8 g/l of the polymer with a cell dry weight of 10.3 g/l

after 60 hrs of cultivation (Vo et al., 2007). *P. putida* GP01 cells, when incubated in alkaline buffers, secreted 3-hydroxyoctanoate and 3-hydroxyhexanoate at constant high pH, the organism produced 3-hydroxyoctanoate and 3-hydroxyhexanoate more efficiently than by incubation without pH control (Wang et al., 2007).

The sludge-isolated *P. aeruginosa* MTCC 7925 demonstrated good capability to synthesize SCL-LCL-PHA co-polymer from unrelated carbon sources. Supplementation of ethanol under N-deficiency boosted the co-polymer yield up to 69% of dry cell weight, which is significantly higher when compared with other SCL-MCL-PHA co-polymer accumulating bacterial species. Stationary PHA culture of *P. aeruginosa* MTCC 7925 was subjected to various doses of different carbons and, N and P deficiencies. Enhanced co-polymer yield was recorded under ethanol- and glucose-supplemented cultures. Interaction of ethanol with N-deficiency boosted co-polymer accumulation maximally (Singh and Mallick, 2008).

The bacterium *P. guezennei* produces a novel PHA mcl with elastomeric properties. This micro-organism, on the basis of the phenotypical features and genotypic investigations can be clearly assigned to the *Pseudomonas* genus and the name of *Pseudomonas guezennei* is proposed. Optimal growth occurs between 33 and 37°C, at a pH between 6·4 and 7·1 and at ionic strength of 15 g l^{-1} of sea salts. The G+C content of DNA is 63·2%. Under laboratory conditions, this bacterium produced a novel, medium-chain-length PHA, mainly composed of 3-hydroxydecanaote (64 mol.%) and 3-hydroxyoctanoate (24 mol.%) (GC-MS, NMR) from a single nonrelated carbon substrate, i.e. glucose (Simon et al., 2008).

Bacillus spp.

Bacillus megaterium has been discovered as the new class IV PHA synthase type example. It largely resembles class III PHA synthase but, *PhaE* is replaced by *PhaR* a 22-kDa protein with no homology to *PhaE*. The *PhaR I* and *PhaC* from *B.megaterium* were localized to inclusion bodies in living cells (McCool and Cannon, 2001). Polymers extracted from *B. megaterium* grown on nitrogen free medium were a complex mixture of beta-hydroxy acids with chain lengths between four to eight carbons (Findlay and White, 1983). *Bacillus* sp. JMa5 isolated from molasses contaminated soil was able to grow at a temperature as high as 45°C and in 250 g/l molasses. The optimum temperature and molasses concentration for PHB production were 35-37°C and 210 g/l respectively. The strain was able to accumulate 25-35% (w/w) PHB during fermentation (Wu et al., 2001). *Bacillus cereus* UW85 when grown on ε-caprolactone, or ε-caprolactone and glucose as carbon sources under nitrogen-limited conditions produced P (3HB) copolymers. When ε-caprolactone was used as a carbon substrate, the bacterial strain produced tercopolymer with 3HB, 3HV and 6HHx units. However, when caprolactone and glucose were supplied together, only homopolymer of P (3HB) was produced (Labuzek and Radecka, 2001).

B. megaterium strain produced a maximum of 40.8 mg, and 39.9 mg cell dry matter PHB, when grown on medium containing cane molasses and glucose as sole carbon sources respectively. Corn steep liquor as alternate cheap nitrogen substrate was best for PHB production apart from ammonium chloride, ammonium sulphate, ammonium oxalate or ammonium phosphates, which were also tested as nitrogen sources (Gouda et al., 2001).

Vazquez *et al.* (2003) reported, about the association of PHB accumulation and phosphotransbutyrylase expression in *B. megaterium.* The complete molecular analysis of genomic regions of the bacterium revealed the presence of a gene coding for the enzyme phosphotransbutyrylase (Ptb). *Ptb* expression was repressed by glucose and activated by the branched amino acids, isoleucine and valine.

Bacillus has been grown on various versatile substrates from glycerol to molasses, producing PHA ranging from 24% to 89% cell dry weight. Molasses is an alternative cheap substrate, which can be used for PHA production by *Bacillus* species. *Bacillus* strain CL1 produced 90% of cell dry mass as PHA from various sugars including raffinose, and did so without requiring a nutrient limitation. The strain utilized Soya molasses to produce a highest cell dry weight of the polymer (Full et al., 2006).

The PHA yields obtained were found to be 13·4, 38, 13·15 and 33·33% CDW for potassium, nitrogen, sulphur and phosphorus limitations, respectively. Gas chromatography–mass spectrometry analysis of the isolated polymers showed the presence of P (3HB) under nitrogen, sulphur and phosphate-limiting conditions and P(3HB-3HV) copolymer under potassium limiting conditions. This ability of *B. cereus* SPV to accumulate different PHA monomers from structurally unrelated carbon sources led to an interest in the molecular analysis of PHA biosynthesis in this organism. To achieve this, PCR was used to identify the polyhydroxy-alkanoate biosynthetic genes in *B. cereus* SPV. Sequence analysis of the PCR products from *B. cereus* SPV revealed the sequence of the putative biosynthetic genes, and possible regions involved in substrate binding (Valappil et al., 2008).

Wang *et al.* (2006), cloned *phaC1* and *phaC1AB* from *Pseudomonas aeruginosa* and *Ralstonia eutropha* into *Bacillus subtilis* DB104. The recombinant strains produced mcl-PHA which was found to be hydroxybutyrate-co-hydroxydodecanoate polymer.

Aeromonas hydrophila

Aeromonas hydrophila 4AK4 normally produces copolyesters (PHBHHx) consisting of 3-hydroxybutyrate (C4) and 3-hydroxyhexanoate (C6). Wild type and recombinant *A. hydrophila* 4AK4 (pSXW02) expressing *vgb* and *fadD* genes encoding *Vitreoscilla* haemoglobin and *Escherichia coli* acyl-CoA synthase respectively, were found able to produce homopolyester poly(3-hydroxyvalerate) (PHV) (C5) on undecanoic acid as a single carbon source. The recombinant grew to 5.59 g/L cell dry weight (CDW) containing 47.74 wt% PHV in shake flasks when growth was conducted in LB medium and PHV production in undecanoic acid. The cells grew to 47.12 g/L CDW containing 60.08 wt% PHV in a 6 L fermentor study. Physical characterization of PHV produced by recombinant *A. hydrophila* 4AK4 (pSXW02) in fermentor showed a weight average molecular weight (M_w) of 230,000 Da, a polydispersity of 3.52, a melting temperature of 103 °C and a glass transition temperature of "15.8 °C. The degradation temperature at 5% weight loss of the PHV was around 258 °C (Xiao-Wen Shen et al., 2009).

Polyhydroxyalkanoate synthase gene *phaC$_{ah}$* in *Aeromonas hydrophila* strain 4AK4 was deleted and its function was replaced by *phaC1$_{ps}$* cloned from *Pseudomonas stutzeri* strain 1317 which favors 3-hydroxyhexanoate (3HHx) and longer chain length monomers. Genes *fadD* and *fadL* encoding *Escherichia coli* acyl-CoA synthase and *Pseudomonas putida* KT2442 fatty acid

transport protein, respectively, were introduced into the recombinant with new $phaC1_{ps}$. Accumulation of a series of novel medium-chain-length polyhydroxyalkanoates (mcl-PHA) consisting of 80-94 mol% 3HHx were observed. The recombinant accumulated 54% poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) in cell dry weight consisting of 94.5 mol% 3HHx or 51% poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) consisting of 82 mol% 3HHx and 16 mol% of 3HO during a two-step cultivation process under nitrogen limitation when grown on sodium hexanoate or sodium octanoate. The two polyesters containing high percentage of 3HHx are physically characterized. They could be used as biodegradable pressure sensitive adhesives, coatings, polymer binding agents in organic-solvent-free paints or a source for chiral *R*-3-hydroxyhexanoate (Jia Jian et al., 2010).

Methylobacterium

Methylotrophic (methanol-utilizing) bacteria offer great potential as cell factories in the production of numerous products from biomass-derived methanol. Bio-methanol is essentially a non-food substrate, an advantage over sugar-utilizing cell factories. Low-value products as well as fine chemicals and advanced materials are envisageable from methanol. For example, several methylotrophic bacteria, including *Methylobacterium extorquens*, can produce large quantities of the biodegradable polyester polyhydroxybutyric acid (PHB), the best known polyhydroxyalkanoate (PHA). With the purpose of producing second-generation PHAs with increased value, we have explored the feasibility of using *M. extorquens* for producing functionalized PHAs containing C-C double bonds, thus, making them amenable to future chemical/biochemical modifications for high value applications.

M. extorquens ATCC 55366 was found unable to yield functionalized PHAs when fed methanol and selected unsaturated carboxylic acids as secondary substrates. However, cloning of either the *phaC1* or the *phaC2* gene from *P. fluorescens* GK13, using an inducible and regulated expression system based on cumate as inducer (the cumate switch), yielded recombinant *M. extorquens* strains capable of incorporating modest quantities of C-C double bonds into PHA, starting from either C6= and/or C8=. The two recombinant strains gave poor results with C11=. The strain containing the *phaC2* gene was better at using C8= and at incorporating C-C double bonds into PHA. Solvent fractioning indicated that the produced polymers were PHA blends that consequently originated from independent actions of the native and the recombinant PHA synthases (Philipp Höfer et al., 2010).

Recombinant *Methylobacterium extorquens* ATCC 55366 harboring *phaC2* from *Pseudomonas fluorescens* GK13 (*M. ex-phaC2*) was capable of producing blends of polyhydroxyalkanoates. By co-feeding methanol and 5-hexenoic acid (C6=), functionalized PHAs (4 ≤ chain length ≤ 6) containing C–C double bonds were created. Bioreactor studies revealed negative impacts of 5-hexenoic acid on biomass and PHA production by reducing overall yields. In contrast, there was a positive relationship between 5-hexenoic acid supply and abundance of polymeric functional groups, i.e., molar portions of monomeric units bearing double bonds increased with increased 5-hexenoic acid supply. Correlation of C6 consumption to methanol addition resulted in a model that allowed for on-line estimation of toxic co-substrate concentration. The functionalized PHAs were ductile and showed signs of side chain

crosslinking, resulting in reduced degrees of crystallinity. Incorporation of 3-hydroxyhex-5-enoate and 3-hydroxyhexanoate into the polymeric chains produced desirable thermal properties with enhanced thermal stability and reduced melting temperatures (139-168 °C). Thermal degradation and melting temperatures obtained suggest a comfortable range for melt-processing of these polymers and allow for autoclaving as convenient sterilization process. Consequently, functionalized PHAs produced in this study are candidates for medical applications as part of biocomposite materials. The use of methanol as main substrate for cultivation of recombinant *M. extorquens* offers the possibility to reduce production costs and develop new process control strategies (Philipp Höfer et al., 2011).

Corynebacterium glutamicum

Lipopolysaccharides free P[3-hydroxybutyrate (3HB)-*co*-3-hydroxyvalerate (3HV)] production was achieved using recombinant *Corynebacterium glutamicum* harboring polyhydroxyalkanoate (PHA) biosynthetic genes from *Ralstonia eutropha*. Cells grown on glucose with feeding of propionate as a precursor of 3HV unit accumulated 8–47 wt% of P(3HB-*co*-3HV). The 3HV fraction in the copolymer was varied from 0 to 28 mol% depending on the propionate concentrations (Ken'ichiro Matsumoto et al., 2011).

Lactococcus lactis

Many bacteria are naturally capable of accumulating biopolyesters composed of 3-hydroxy fatty acids as intracellular inclusions, which serve as storage granules. Recently, these inclusions have been considered as nano-/microbeads with surface-attached proteins, which can be engineered to display various protein-based functions that are suitable for biotechnological and biomedical applications. In this study, the food-grade, generally-regarded-as-safe gram-positive organism *Lactococcus lactis* was engineered to recombinantly produce the biopolyester poly(3-hydroxybutyrate) and the respective intracellular inclusions. The codon-optimized polyhydroxybutyrate biosynthesis operon *phaCAB* from *Cupriavidus necator* was expressed using the nisin-controlled gene expression system. Recombinant *L. lactis* accumulated up to 6% (wt/wt) poly(3-hydroxybutyrate) of cellular dry weight. Poly(3-hydroxybutyrate) granules were isolated and analyzed with respect to bound proteins using biochemical methods and with respect to shape/size using transmission electron microscopy. The immunoglobulin G (IgG) binding ZZ domain of *Staphylococcus aureus* protein A was chosen as an exemplary functionality to be displayed at the granule surface by fusing it to the N terminus of the granule-associated poly(3-hydroxybutyrate) synthase. The presence of the fusion protein at the surface of isolated granules was confirmed by peptide fingerprinting using matrix-assisted laser desorption ionization-time of flight (mass spectrometry). The functionality of the ZZ domain-displaying granules was demonstrated by enzyme-linked immunosorbent assay and IgG affinity purification. In both assays, the ZZ beads from recombinant *L. lactis* performed at least equally to ZZ beads from *Escherichia coli*. Overall, in this study it was shown that recombinant *L. lactis* can be used to manufacture endotoxin-free poly(3-hydroxybutyrate) beads with surface functionalities that are suitable for biomedical applications (Jun Mifune et al., 2009).

Rhizobium meliloti

In a *Rhizobium meliloti* PHB⁻ mutant strain, P (3HB) accumulation was restored to wild type

level by the introduction of a plasmid encoded *R. meliloti phaC* gene (Tombolini et al., 1995).

Paracoccus denitrificans

In *Paracoccus denitrificans*, an additional *phaC* gene on a plasmid doubles the wild-type PHA levels in a pentanol-grown parent strain (Ueda et al., 1996).

METABOLIC ENGINEERING OF PHA BIOSYNTHESIS IN HIGHER ORGANISMS

Yeast

The *phaCRe* gene of the *R. eutropha* was used to construct a yeast plasmid, which enabled expression of the functional synthase enzyme in *Saccharomyces cerevisiae.* These cells accumulated only up to 0.5% of cell dry weight as PHB, with accumulation occurring in the stationary PHA of batch growth. In these recombinant yeast cells, PHB synthesis is catalyzed by native cytoplasmic acetoacetyl-CoA thiolase, a native β-oxidation protein processing D-3HB-CoA dehydrogenase activity and heterologous *pha* synthase. Low levels of β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase were detected. This eukaryotic system probably needs elevation of these activities for enhanced PHB production. *S. cerevisiae* transformed with *Pseudomonas aeruginosa* PHAC1 synthase and modified for peroxisome targeting by addition of the carboxyl 34 amino acids from *Brassica napus* isocitrate lyase produced PHA in their peroxisome. PHA containing even-chain monomers from 6-14 carbons was found in recombinant yeast grown on oleic acid, while odd-chain monomers from 5-15 carbons were found in PHA from recombinant yeast grown on heptadecenoic acid with 0.45 % maximum amount of PHA (Poirier et al., 2001).

Insect cell lines

PHB synthesis by *Spodoptera frugiperda* cell lines has been reported by simultaneous transfection of mutant form of the rat fatty acid synthase and PHA synthase from *R. eutropha*. Approximately 1 mg of PHB was isolated from a one-liter culture of these cells corresponding to 0.16% of cell dry weight. Though not an efficient system, however, provides an example of alternative, eukaryotic enzymes for the generation of P (3HB) intermediates (Williams et al., 1996).

Plants

Plants are interesting targets for expression of PHA biosynthetic genes. Transgenic plants could produce PHA directly from CO_2 and solar energy and at least theoretically at costs which are comparable to those of other biopolymers already obtained from plants. PHA biosynthetic genes have been expressed in Arabidopsis thaliana and in agricultural crops such as *Brassica napus, Gossypium hirsutum, Nicotiana tobaccum, Solanum tuberosum and Zea mays* (John and Keller, 1996; Hahn et al., 1999; Houmiel et al., 1999; Nakashita et al., 1999; and Steinbüchel, 2001).

Short-chain-length/medium-chain-length (SCL/MCL) polyhydroxyalkanoate (PHA) was produced in the plastids of *Arabidopsis thaliana*. Phe87Thr (F87T) mutated 3-ketoacyl-acyl carrier protein (ACP) synthase III (FabH) from *Escherichia coli*, and Ser325Thr/Gln481Lys (ST/QK) mutated polyhydroxyalkanoate (PHA) synthase (PhaC1) from *Pseudomonas* sp. 61"3, along with the β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) from *Ralstonia eutropha* (*Cupriavidus necator*) genes were introduced into Arabidopsis. The transgenic Arabidopsis produced PHA copolymers composed of monomers consisting of 4-14 carbons. The introduction of the engineered PHA

synthase resulted in a 10-fold increase in PHA content compared to plants expressing the wild-type PHA synthase. In addition, expression of the engineered *fabH* gene in the plastid led to an increase in the amount of the SCL monomer, 3-hydroxybutyrate, incorporated into PHA, and contributed to supply of MCL monomers for PHA production (Ken'ichiro Matsumoto et al., 2009).

Synthesis of PHA in plants was initially explored by the expression of PHA biosynthetic genes of the bacterium *Cupriavidus necator* in the well-studied plant *Arabidopsis thaliana*. But PHB accumulation of only 0.1% of the plant dry weight was achieved. Moreover, the growth of the transgenic plants was severely reduced probably due to depletion of one or more essential substrates for growth (Poirier et al., 1992). To overcome this problem, PHB biosynthetic pathway was targeted to the plastids of *A. thaliana* through an N-terminal transit peptide. This resulted in PHB accumulation of up to 14% of the dry weight with no deleterious effects on plant growth (Nawrath et al., 1994). Recently all three genes necessary for PHB biosynthesis were transformed to *A. thaliana* in a single transformation event. These plants accumulated more than 4% of their fresh weight (approximately 40% of their dry weight) of PHB in leaf chloroplasts. Accumulation of high levels of PHB in transgenic *A. thaliana* plants were not accompanied by any appreciable change either in the composition or the amount of fatty acids. Substantial changes were, however, observed in the levels of various organic acids, amino acids, sugars and sugar alcohols (Bohmert et al., 2000). Synthesis of msc-PHA in *A. thaliana* was explored with the transformation of *phaC1Pa from Pseudomonas aeruginosa* modified for peroxisome targeting by addition at the carboxyl

end of the protein with 34 amino acids from the *Brassica napus* isocitrate lyase. Transgenic *A. thaliana* accumulated approximately 4 mg of msc-PHA per g of dry weight. The plant PHA contained saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6-16 carbons with 41% of the monomers being 3-hydroxyoctanoic acid and 3-hydroxyoctenoic acid. This achievement indicates that the β-oxidation of plant fatty acids can generate a broad range of saturated and unsaturated (R) – hydroxyalkanoate monomers that can be used to synthesize msc-PHA. *Arabidopsis* and *Brassica* were engineered to produce PHBV in leaves and seeds respectively, by transforming threonine deaminase gene from *E. coli* and PHB biosynthetic genes from *Cupriavidus necator* (Mittendorf et al., 1998).

Polyhydroxybutyrate [P(3HB)] was produced in the transgenic tobacco harboring the genes encoding acetoacetyl-CoA reductase (PhaB) and polyhydroxyalkanoate synthase (PhaC) from *Ralstonia eutropha* (*Cupriavidus necator*) with optimized codon usage for expression in tobacco. P(3HB) contents in the transformants (0.2 mg/g dry cell weight in average) harboring the codon-optimized *phaB* gene was twofold higher than the control transformants harboring the wild-type *phaB* gene. The immunodetection revealed an increased production of PhaB in leaves, indicating that the enhanced expression of PhaB was effective to increase P(3HB) production in tobacco. In contrast, codon-optimization of the *phaC* gene exhibited no apparent effect on P(3HB) production. This result suggests that the efficiency of PhaB-catalyzed reaction contributed to the flux toward P(3HB) biosynthesis in tobacco leaves (Ken'ichiro Matsumoto et al., 2011).

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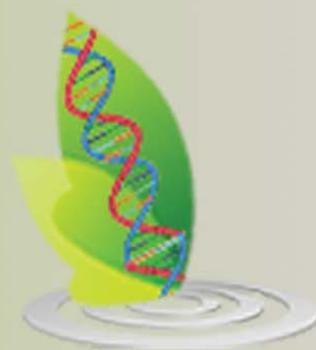
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