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ORIGINAL PAPER

Bioproduction of polyhydroxyalkanoates from bacteria: a metabolic approach

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Abstract The advent of molecular biological techniques and a developing environmental awareness initiated a renewed scientific interest in Polyhydroxyalkanoates (PHAs) and the biosynthetic machinery for PHA metabolism has been the area of research over the last two decades. PHAs are polyesters of hydroxyalkanoates synthesized by numerous bacterial species with atleast five different PHA biosynthetic pathways. These are accumulated as an intracellular carbon and energy storage material. This diversity, in combination with genetic and molecular engineering has opened up this area for development of optimum PHA producing organisms. Even though PHAs have been recognized as a good candidate for biodegradable plastics, their industrial application is limited owing to high production cost. The classical microbiology and modern molecular biology have been brought together to decipher the intricacies of PHA metabolism both for production purposes and for the unraveling of the natural role of PHA. This review provides an overview of the different PHA biosynthetic systems, the enzymes involved in PHA biosynthesis and there genetic background followed by a detailed summation of how this natural diversity is being used to develop commercially attractive recombinant process for large scale production of PHAs.

Keywords Biodegradable plastic · Polyhydroxybutyrate · Metabolic engineering · Vector · CAB operon

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Introduction

Synthetic plastics are resistant to degradation and consequently their disposal is fuelling an international drive for the development of biodegradable polymers. Biodegradable plastics are polymers, which undergo degradation particularly by enzymes into CO_2 and H_2O under aerobic conditions and into methane and inorganic compounds under anaerobic conditions. The same can be measured by standardized tests, in a specified time period, reflecting available disposable conditions.

Biodegradable plastics are largely divided into three categories:

(1) Chemically synthesized polymers: These are susceptible to microbial attack and can't be used as commercial plastics because their properties like biodegradability and melting point differs e.g. Polyglycollic acid, poly (e-caprolactone), polyvinyl alcohol etc.
(2) Starch based biodegradable plastics: These polymers are partially degradable because starch particals act as a linkage between the plastic matrix and are the sites of enzyme action and the residue left after degradation are recalcitrant (Khanna and Srivastava 2005).
(3) Polyhydroxyalkanoates (PHA): These are aliphatic polyesters of hydroxyalkanoates, similar to conventional plastics and are naturally produced via a microbial process on nutrient limiting medium (nitrogen and phosphorus) but with a surplus of sugar which serve as carbon and energy source.

The general structure of PHA consist of 3-hydroxy fatty acid monomers arranged in head to tail manner i.e. the carboxylic group of one monomer form an ester bond with the hydroxyl group of the neighboring monomers. (Fig. 1)

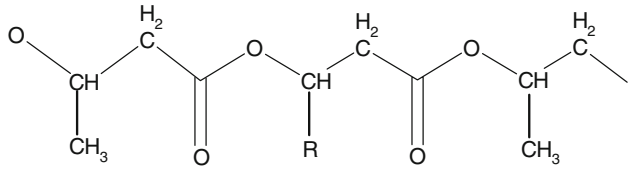

Fig. 1 Structure of PHA. n varies from 600 to 35,000, R=hydrogen Poly(3-hydroxypropionate)/methyl Poly (3-hydroxybutyrate) to nonyl Poly (3-hydroxydodecanoate)

The hydroxyl substituted carbon atom is of R-configuration when it shows chirality. At C-3 or β-position an aromatic/unsaturated/halogenated/epoxidized/branched alkyl group is present. Substitutions in this alkyl side chain leads to formation of a new polymer e.g. incorporation of Cynophenylvalerate and 4, 5, 6 hydroxy acid results in formation of a diverse PHA polymer family (Madison and Huisman 1999).

Depending upon the number of carbon atom present in the side chain, PHAs are divided mainly into two groups:

(A) Short side chain (ssc)
(B) Medium side chain (msc)

Short side chain (ssc) consists of 3–5 carbon atoms whereas 6–14 carbon atoms are present in medium side chain (msc) (Anderson and Dawes 1990). The difference lies in the substrate used and specificity of the enzyme PHA synthase to accept hydroxyalkanoates to a certain range. The ssc is produced by polymerization of 3-hydroxyalkanoate and msc is produced by polymerization of acetyl CoA (Steinbuchel 1991; Steinbuchel and Hein 2001).

Current problems in PHA Production

The major obstacle for the commercial production of PHA is the high cost of sustainable biotechnological process, which includes the product yield, the process of final product separation and the equipment costs relative to petroleum derived plastics (Sudesh et al. 2000).

PHAs can be recovered and purified from biomass by a number of different techniques mostly, which involves mechanical (heat treatment) and chemical (cell disruption) followed by chemical (hypochlorite) and enzymatic treatment. Although these methods are applicable in small-scale fermentation systems, there are drawbacks for large-scale production of PHAs since hypochlorite causes limited hydrolysis of PHA while heat treatment and use of enzyme cocktails are costly (Zhuang et al. 1999). One of the major drawbacks for the commercial PHA production is to maintain the special growth conditions required for PHA synthesis usually unbalanced nutrient conditions that cause slow growth (Byrom 1987). Also additional feeds added in the fermentor, add to the cost as they expand the infrastructure and impose additional quality control.

Monomer precursors are added in very controlled manner for the stable PHA synthesis in recombinant *E. coli*. The expression of all the genes involved in the pathway should be adequate and have to be expressed from extrachromosomal DNA such as in plasmid and therefore depend upon high copy number and high expression level. But for large-scale fermentation, maintenance of the plasmid and stable expression are the major drawbacks (Huisman et al. 2006).

Even though the price of the PHA is still too high, current advances in fermentation and purification technology as well as the development of superior bacterial strains by recombinant DNA technology are likely to lower the price of PHA. The isolation and development of bacterial strains that can utilize cheap carbon substrates should be opted intensively, but eventually metabolic engineered bacterial strains may likely surpass the wild type bacteria currently in use.

Polyhydroxyalkanoates biosynthesis

The most extensively produced microbial bioplastics are Polyhydroxyalkanoates (PHA), Polyhydroxybutyrate (PHB) and their derivatives. Much other polyester can also be produced by microorganisms and most of them utilize similar biosynthetic machinery. However these polyesters lacks large-scale applications (Ha and Cho 2002) and hence this review concentrates only about PHAs and PHBs.

There are four different pathways for PHA synthesis (Steinbuchel 1991), which consist of three enzymatic reactions catalyzed by successive action of β-Ketoacyl-CoA thiolase (*phbA*), acetoacetyl CoA (*phbB*), P(3HB) polymerase (*phbC*) (Madison and Huisman 1999). These different pathways are explained below with respect to the different microorganisms in which they are found.

Biosynthetic pathway in *Ralstonia eutropha* (*Alcaligenes eutrophus*)

In *R. eutropha*, metabolism of carbohydrates leads to the biosynthesis of PHA. The first reaction involves condensation of two acetyl Coenzyme A (acetyl CoA) molecules into acetoacetyl CoA by β-Ketoacyl-CoA thiolase or β-Ketothiolase enzyme, encoded by *phbA* gene. In the second step the stereospecific reduction of acetoacetyl CoA to R-3-hydroxybutyryl CoA by an NADPH dependent acetoacetyl CoA dehydrogenase or reductase, encoded by *phbB* gene takes place. The final reaction involves the polymerization or incorporation of R-3-hydroxybutyryl CoA enantiomer into poly 3-hydroxybutyrate by P(3HB)

polymerase or PHB synthase, encoded by *phbC* gene (Anderson and Dawes 1990; Cevallos et al. 1996) (Fig. 2).

Biosynthetic pathway in *Rhodopseudomonas rubrum*

This is the second type of pathway, which is found in *Rhodopseudomonas rubrum*. In *R. rubrum*, β-oxidation of fatty acid leads to the biosynthesis of PHA. The first reaction remains the same as that for *R. eutropha* leading to the formation of acetoacetyl CoA, which is then reduced into L-(+)-3-hydroxybutyryl CoA by a NADH dependent reductase. In the final step L-(+)-3-hydroxybutyryl CoA is converted into D-(−)-3-hydroxybutyryl CoA by two enol CoA hydratase enzymes (Khanna and Srivastava 2005) (Fig. 3).

Biosynthetic Pathway in *Pseudomonas* group I

This is the third type of PHA biosynthetic pathway, which is found in most *Pseudomonas* species, belonging to rDNA homology group-I e.g. *P. oleovorans*. In these organisms fatty acid β-oxidation of alkanes, alkanols or alkanoic acids leads to the synthesis of medium side chain (msc) PHAs.

Biosynthetic pathway in *Pseudomonas* group II

This is the fourth type of PHA biosynthetic pathway found in *Pseudomonas* belonging to rDNA homology group-II. Synthesis of PHA results from Denovo fatty acid synthesis pathway which involves the synthesis of copolymers of medium side chain (msc) 3-hydroxyalkanoates (3HA) from acetyl CoA (Fig. 4).

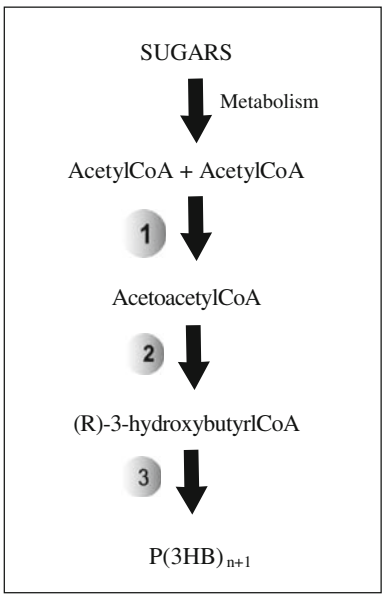

Fig. 2 Biosynthesis of PHA from carbohydrates in *R. eutropha*. (1) (-Ketothiolase, (2) NADPH dependent acetoacetyl CoA reductase, (3) P(3HB) polymerase or synthase

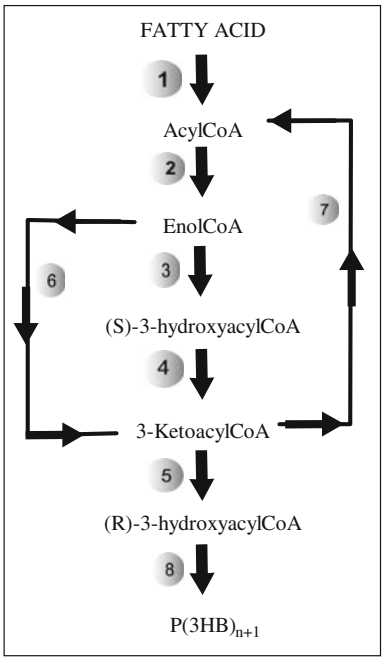

Fig. 3 Biosynthesis of PHA from fatty acid (-oxidation in *R. rubrum*. (1) Acyl CoA ligase, (2) Acyl CoA dehydrogenase, (3) Enol CoA hydratase, (4) 3-hydroxyacyl CoA dehydrogenase, (5) 3-Ketoacyl CoA reductase, (6) R-enolCoA hydratase, (7) 3-Ketothiolase, (8) PHB synthase

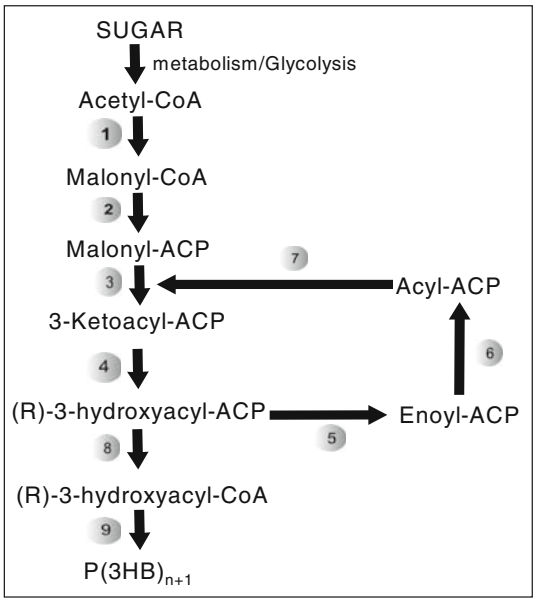

Fig. 4 Biosynthesis of PHA from "denovo fatty acid synthesis" in *pseudomonas* group-II. (1) Acetyl CoA Carboxylase, (2) ACP malonyl transferase, (3) 3-Ketoacyl ACP-Synthase, (4) 3-Ketoacyl ACP reductase, (5) 3-Hydroxyacyl ACP reductase, (6) Enol ACP reductase, (7) 3-ketoacyl ACP synthase, (8) 3-hydroxy acyl ACP-CoA transferase, (9) PHB synthase

Genetic level studies

The organization of the genes and enzymes involved in the biosynthesis of PHA varies from organism to organism. Due to the increasing diversity of PHA biosynthetic pathways, it is not surprising that the PHB loci have diverged considerably. The loci encoding the genes for PHA formation have been characterized from 18 different species.

In *Alcaligenes latus*, *Pseudomonas acidophila*, and *Ralstonia eutropha* the *phbCAB* genes are arranged tandemly on the chromosome, whereas in *Acinetobacter* species the *phbCAB* gene are not present in the same order, however all of them consist of complete CAB operon (Peoples and Sinskey 1989; Umeda et al. 1998) (Fig. 5a).

In *Zoogloea ramigera*, *Paracoccus denitrificans* and *Rhizobium meliloti* the *phbC* and *phbAB* loci are unlinked or interrupted i.e. *phbA* and *phbB* together form an operon whereas *phbC* is located elsewhere in the chromosome (Lee et al. 1996; Tombolini et al. 1995) (Fig. 5b).

In *Chromatium vinosum*, *Thiocystis vinolaceae* and *Synechocystis* sp. two genes *phbE* and *phbC* encodes the two-subunit enzyme PHA polymerase. *phbEC* and *phbAB* are present in one loci but there orientations are opposite (Hein et al. 1997; Liebergesell and Steinbuchel 1992) (Fig. 5c).

In *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli* and *Rhodococcus ruber* an additional gene *phbP* is present with an unknown function in the PHB regulation. Here also *phbCP* and *phbAB* are present in the same locus but with different orientations therefore only the PHB polymerase encoding gene *phbC* has been identified so far (Hustede and Steinbuchel 1993) (Fig. 5d).

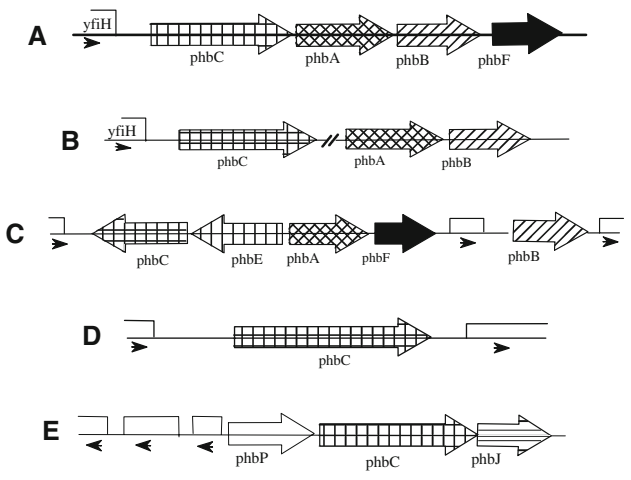

Fig. 5 (**a**) Complete phbCAB operon in *P. acidophila*; (**b**) Interrupted *phb* loci in *Z. ramigera*; (**c**) *phb* loci from organisms that encode two subunit P(3HB) polymerases in *C. vinosum*; (**d**) Incomplete *phb* loci in *M. extorquens*; (**e**) The *phbCJ* locus of *A. caviae*

In *Aeromonas caviae* the PHB polymerase encoding gene *phbC* has a flanking gene *phbJ*, which provides monomer for the enzyme (Fukui et al. 1998) (Fig. 5e).

In *Pseudomonas oleovorans* and *P. aeruginosa* the phb loci is having two *phbC* gene i.e. *phbC1* and *phbC2*. These two genes are separated by an additional gene *phbZ*, which encodes intracellular PHA depolymerase enzyme. The enzymes encoded by *phbC1* and *phbC2* are identical in their primary structure and have almost similar substrate specificity (Huisman et al. 1991; Timm et al. 1990).

In *C. vinosum*, *P. acidophila*, *R. eutropha*, *R. meliloti* and *T. violacea* an additional gene *phbF* is present with an unknown function in PHA regulation (Povolo et al. 1996). In *P. acidophila*, *R. eutropha* and *Z. ramigera* have a gene present upstream of phbCAB operon and which is identical to the hypothetical *E. coli* protein YfiH.

During the course of evolution, *phbC* gene was sometimes arranged with genes that supply monomers like *phbAB* or *phaJ* and sometimes with genes involved in PHA regulation like *phbZ*. Evolutionary forces resulted in the clustering of *phb* genes in an operon with same transcriptional units as in *P. acidophila*, *R. eutropha*, *Acinetobacter*, *A. latus*, *A. caviae* and at times in separate transcriptional units as in *Z. ramigera*, *P. denitrificans*, *R. meliloli*, *C. vinosum*, *T. violaecae*, *P. oleovorans*, *P. putida* etc. On the other hand some resulted in diversely oriented additional or flanking *phbF* and *phbP* genes. In *C.vinosum* and *T.violacea* the *phbEC* gene have neighboring *phbA* and *phbB* genes whereas these genes are not present in *Synechocystis*. Therefore in the course of evolution fusion of *phbEC* or splicing of *phaC* may result the rearrangements in the phb loci.

Approaches to metabolic engineering

To realize sustainable industrial processes based on the use of PHA biopolyester, the high manufacturing cost needs to be reduced by establishing an efficient recombinant production system of PHA with desired properties. The strains used for general cloning works and gene expression have been summarized in Table 1.

Construction of recombinant plasmid from *Aeromonas caviae*

Aeromonas caviae involves three structural genes for PHB biosynthesis, $phaC_{AC}$ encoding PHA synthase, $phaJ_{AC}$ encoding (R)-specific enoyl CoA hydratase and $phaP_{AC}$ encoding a granule associated protein (Fukui et al. 2001).

Plasmid pBSEE32phbAB was constructed for PHB and PHA copolymer biosynthesis. The plasmid vector pBSEE32 was constructed by ligation of *Eco*RI digested pJRDEE32, a

Table 1 Description of strains and plasmids used for general cloning and gene expression

Strains/plasmids	Origin	Genotype/phenotype	References
E. coli strains			
JM109		*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 (*lac-proAB*)	Sandoval et al. (2005)
DH5α		*supE*44 *lacU*169 (Φ80*lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sandoval et al. (2005)
XL1-Blue		*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *suppE*44 *relA*1 λ^- *lac* F′[*proAB lacI*^{q} *lacZΔM*15 Tn10 (Tet^r)]	Stratagene[a]
W3110		F^- *mcrA mcrB IN(rrnD rrnE)* 1 λ^-	KCTC[b]
WB101		W3110 (*fadB*::Km)	Park and Lee (2003)
WB108		W3110 (*fadB*::Km *maoC*::Tc)	Park and Lee (2003)
WB112		W3110 (*fadB*::Km *yfcX*::Tc)	Park and Lee (2003)
Plasmids			
pJC7	pBBR233	Amp^r *buk*$^+$ *ptb*$^+$	Cevallos et al. (1996)
pBHR68	pBluescriptSKM	Amp^r *phbC*$^+$ *phbA*$^+$ *phbB*$^+$	Sandoval et al. (2005)
pBHR69	pBluescriptSKM	Amp^r *phbA*$^+$ *phbB*$^+$	Sandoval et al. (2005)
pUCAB	pUC18	Amp^r *phbA*$^+$ *phbB*$^+$	Zhuang et al. (1999)
pKKAB	pKK223	Amp^r *phbA*$^+$ *phbB*$^+$	Gerngross et al. (1994)
pMCS2PTK	pBBR1MCS2	Km^r *buk*$^+$ *ptb*$^+$	Gerngross et al. (1994)
p68CM	pBHR68	Amp^r Δ*phbC phbA*$^+$ *phbB*$^+$	Gerngross et al. (1994)
p68CMPTK	pBHR68	Amp^r Δ*phbC phbA*$^+$ *phbB*$^+$ *buk*$^+$ *ptb*	Gerngross et al. (1994)
pTG01		Km^r *phbA*$^+$ *phbB*$^+$	Zhuang et al. (1999)
$pLGP_P$		Amp^r *phaG*$^+$	Zhuang et al. (1999)
P10499A	pTrc99A	*gntT*104 promoter; Ap^r	Park and Lee (2003)
PMCS104613C2	pBR1MCS	*gntT*104 promoter, $phaC2_{Ps}$	Park and Lee (2003)

[a] Stratagene cloning system

[b] Korean collection for type cultures, Republic of Korea

fragment of *A. caviae* gene containing polycistronic genes for $phaP_{AC}$ (granule associated protein), $phaC_{AC}$ ((R)- specific enoyl-CoA hydratase) with promoter $Ppha_{AC}$ and *Eco*RI site of Bluescript II SK(+). The resultant shuttle digested *R. eutropha* H16 fragment pBS-$phbAB_{RE,}$ containing genes for $phaA_{RE}$ (*β*-ketothiolase) and $phaB_{RE}$ (NADPH dependent acetoacetyl CoA reductase) which results in recombinant plasmid pBSEE32phbAB. For accumulation of P(3HB-co-3HHx) copolyester from dodecanoate, *E. coli* LS5218[fadR601 ato C(Con)] was used as the host strain (Fig. 6).

An increase in the copolymer fraction were observed for mutants (16–18 mol%) compared to the wild type (10 mol%). Highly active mutants E2-50 and T3-11 of a synthase that is a key enzyme essential for bacterial synthesis of biodegradable polyester, polyhydroxyalkanoate (PHA) have been successively acquired. These mutants exhibited a 6.5- and 3-fold higher PHA productivity as compared to the wild type. There is a biphasic pattern of the relationship between enzyme activity toward 3HB-CoA and PHB content in dry cells, keeping linearity up to a PHB content of ca. 45 wt.% and then gradually reaching a

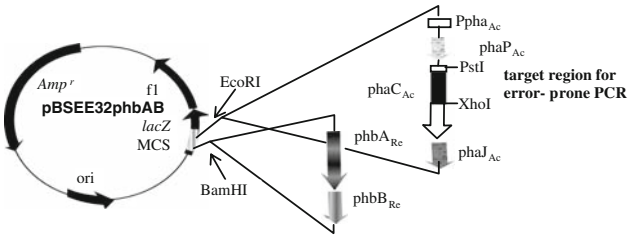

Fig. 6 Plasmid vector pBSEE32phbAB used for synthesizing PHA

plateau corresponding to a content of ca. 55 wt.%. This suggests that insufficient monomer substrate is supplied for the wild-type and for mutants that exhibit activities exceeding 0.01 U/mg (kichise et al. 2002).

Enhanced PHA production from recombinant *Alcaligenes latus*

The *phbC* gene of *A. latus* was isolated and recombined in an *E. coli A. latus* shuttle vector plasmid pkTC32 and transformed by electroporation into the parent *A. latus* in order to amplify the pHB synthase. The isolated *phbC* gene was ligated to the pSK(+) vector designated as plasmid pAL 32, excised from plasmid PAL32 by *Not* I/*Eco*R I double digestion, ligated into the *E. coli A. latus* shuttle vector plasmid pKT230 and then constructed as a 14.2 kb long recombinant plasmid pkTC32 (Park and Lee 2003).

The enhanced PHB biosynthesis mechanism produced in the transformant *A. latus* was investigated by measuring the variations of enzyme activities related to the PHB biosynthesis. The maximum concentration and content of PHB in *A. latus* increased significantly from 3.1 to 3.7 g/l and from 50.2 to 65%, respectively, after the enforcement of its own *phb*C gene, and the rate of PHB biosynthesis also accelerated significantly. The plasmid stability of the transformant *A. latus* remained around 85%, which was similar to that of the transformant *R. eutropha* even though a low concentration of kanamycin was used as the selective pressure. The reintroduced cloned *phb*C gene had an influence both on the PHB synthase activity and the other two enzyme activities, yet most significantly on the PHB synthase that increased from 3.4 to 4.8 Umg/1 proteins after 30 h. The increased ketothiolase and acetoacetyl-CoA reductase activities may have been due to an accelerated flow of the accumulated intermediates, acetoacetyl-CoA and hydroxybutyryl-CoA in the polymerization of PHB, thereby reducing the product inhibitions as described by Jung and Lee (1997). Accordingly, the amplification of PHB synthase through the transformation of a cloned *phb*C gene into its parent *A. latus* would appear to be an effective strain development method for the overproduction of PHB, as previously observed using the transformant *R. eutropha*.

Accumulation of PHA & PHA-copolymer from genetically manipulated *Pseudomonas* sp.

A recombinant *E. coli* is constructed by co-expression of genes of *β*-Ketothiolase (*phbA*), acetoacetyl-CoA (*phbB*) and 3-hydroxyacyl-ACPCoA transacylase (*phaG*) respectively in *E. coli* DH5*α*. Plasmid pBHR69 having *phbAB* genes was digested with *Pst*I and ligated with pUC18. Digestion of the resulting plasmid pUCAB with *Kpn*I and *Hind*III resulted in a 2.4 kb *phbA* and *phbB* genes fragment, which were ligated into the host vector pBBR1MCS2. The resulting plasmid pPG01 contains *phbA* and *phbB* genes under the *lacZ* promoter P (lac) and kanamycin resistance marker Km(r).

Plasmid pBHR91 was used for the amplification of $phaGp_p$ gene. pBHR91 was digested with *Xba*I and *Hind*III and the resulting plasmid $pLGP_p$ contains the $phaGp_p$ gene under a *lacZ* promoter and ampicillin resistance marker Amp(r).

A recombinant strain of *P. putida* U (*P. putida U Δfad BA-pha Z*) was constructed by using plasmid pKB2 for amplification of 892 bp *phaZ* gene (encoding PHA depolymerase) of *P. putida U*. This was further subcloned into plasmid pBBR2MCS-3, which replicates autonomously in *P. putida U* (pMCZ) and was cloned into the hyper expressed vector pQE32 resulting in pQE Depol (Sandoval et al. 2005).

The amount of 3-hydroxyl polyhydroxyalkanoate derivatives accumulated by this recombinant strain were: 1.77 g/l (2.25 g/g cell dry weight (cdw)) of 3-OH-6-phenylhexanoic acid; 1.93 g/l (2.45 g/g cdw) of a mixture of 3-OH-5-phenylvaleric acid (30%) and 3-OH-7-phenylheptanoic acid (70%) and 1.92 g/l (2.44 g/g cdw) of a mixture containing 3-OH-6-phenylhexanoic acid (35%) and 3-OH-8-phenyloctanoic acid (65%). These data indicate that the bioconversion yield of n-PhAs into 3-OH-PhAs using the genetically manipulated strain *P. putida ΔfadBA-phaZ* is higher (85, 87, and 81%, respectively) than the wild strain (80%).

Overproduction of PHA from Recombinant *R. eutrophus*

The pBBr1MCS-2 vector (5.52 kb) was linearized at the *Eco*RI/*Bam*HI site. *P. oleovorans PhaC1* excised from pUC19-CAB3 was ligated to pBBr1MCS-2 vector, by digesting both the vectors with *Eco*RI/*Bam*HI enzymes resulting in the 7.22 kb plasmid pBBr1MCS2$phaC_{po}$. First two rounds of site directed mutagenesis were performed on $phaC_{po}$ to introduce two unique restriction sites *Mfe*I and *Mlu*I at nucleotides 673 (Ile 225) and 1207 (Arg 403), respectively.

The primers *phaC* FS-A and *phaC* FS-D contained a *Psh*A1 and a *Dra*III site, respectively, and therefore, the

site directed mutagenized *phaC1*$_{po}$ (1.2 kb), containing *Mfe*I and *Mlu*I sites, was cloned into the *Psh*AI/*Dra*III-digested pBBr1MCS-2 *phaC*$_{po}$, resulting in the 7.22 kb pBBr1MCS-2 *phaC*$_{po}$-III plasmid (pBBr1MCS-2 *phaC*$_{po}$I225L). To construct pBBr1MCS-2 *phaC*$_{po}$I225L-Linker, a central 540 bp portion of the *phaC1*$_{po}$ gene was removed by *Mfe*I and *Mlu*I. A 20 bp nucleotide linker flanked by *Mfe*I and *Mlu*I restriction sites was digested with same restriction enzymes and ligated into pBBr1MCS-2 *phaC*$_{po}$I225L, resulting in the 6.7 kb plasmid pBBr1MCS-2 *phaC*$_{po}$I225L-Linker. The pBBr1MCS-2 *phaC*$_{po}$I225L-Linker gene was expressed under the control of the phb$_{R.eutropha}$ promoter (Niamsiri et al. 2004).

The recombinant *R. eutrophus* expressing either wild-type *phaC1*$_{po}$ or mutant I225L *phaC1*$_{po}$ produced 4.2 g/g PHA content per dcw. The PHAs produced by the I225L PhaC1$_{po}$ mutant consisted mainly of 3-hydroxyocanoate (3HO) (86 mol%) and 3-hydroxyhexanoate (3HHx) (12 mol%), with a partial molar fraction of 3-hydroxybutyrate (3HB) (2 mol%) and traces of 3-hydroxydecanoate and 3-hydroxydeodecanoate. The composition was similar to that of the PHA from wild-type PhaC1$_{po}$. Differential scanning calorimetry (DSC) showed that both polymers are copolymers and not blends, since both have single (sharp) *Tg* and *Tm* values. *Tg* and *Tm* were −31 and 54°C, respectively, for PHA from the wild-type PhaC1$_{po}$ and 30 and 55°C, respectively, for PHA from the I225L PhaC1$_{po}$.

Construction of transformant *E. coli* by PCR mediated mutagenesis, site-specific saturation mutagenesis and in-vitro recombination

E. coli JM109 was used as a host strain for screening mutants of *Pseudomonas* sp. 61-3 PHA synthase (*phaC1*$_{ps}$) and for P(3HB) accumulation. The plasmid vector, pGEM″phaC1AB was constructed for the biosynthesis of P(3HB) in *E. coli* JM109 strain.

First the plasmid vector pGEM′phbCAB$_{Re}$ carrying the *Ralstonia eutropha* P(3HB) operon with *Csp* 451 and *Pst* I digested its promoter and terminator region and the resulting 6.1 kb DNA fragment was ligated with a synthetic *Cso* 451-*Xba*I-*Eco*RI-*Bgl*II-*Pst*I linker, which results in pGEM″Abex vector. Then a plasmid was constructed for the random mutagenesis of the *phaC1*$_{ps}$ gene by PCR-operated addition of restriction sites, *Nde*I and *Bam*HI, within an initiation codon and downstream of a stop codon, respectively. The *phaC1*$_{ps}$ gene (∼1.7 kb) was amplified by using pBSEX22 plasmid (Matsusaki et al. 2000) as a template with primers C1NdeI and C1BamHI. The PCR products was purified and digested with *Nde*I and *Bam*HI, then subcloned into pET-23 a(+), resulting in pETphaC1$_{ps}$ plasmid. DNA sequencing confirms the DNA sequence of *phaC1*$_{ps}$ gene region of pETphaC1$_{ps}$. Finally *Xba*I and *Bam*HI digestion of pETphaC1$_{ps}$ resulting in 1.7 kb fragment containing the *phaC1*$_{ps}$ gene and the Shine-Dalgarao sequence of pET vector, was purified and ligated with *Xba*I and *Bgl*II digested pGEM″Abex. The resulting plasmid, pGEM″phaC1AB, was used for enhanced P(3HB) production and accumulation (Takase et al. 2003).

PCR-mediated mutagenesis showed an improvement in the function of PhaC1Ps in terms of its ability to produce poly(3-hydroxybutyrate) [P(3HB)] in recombinant *E. coli*. Mutants showed two times or more greater P(3HB) content than that (0.1 wt.%) within recombinant *E. coli* cells harboring the wild type *phaC1*Ps. Site-specific saturation mutagenesis showed 13- to 38- fold higher P(3HB) content in mutants as compared to the wild type. Whereas when in vitro recombination performed the P(3HB) contents of all the double mutants were greatly elevated, as much as 340- to 400-fold higher than in the wild type.

Conclusion

Recombinant PHA production will undoubtedly thrive on the enormous biological diversity of nature, where novel protein activities can be obtained from exotic places, while gene cloning becomes less and less of a technological hurdle. The optimization of fermentation systems for these recombinant organisms will also remain a challenge. Since PHAs are not natural products of *E. coli*, the responses by high cell density cultures to nutrient limitations that trigger subsequent feeds are unpredictable. New fermentation feeding strategies will therefore have to be developed.

This review attempts to bring together the genetic engineering and biochemical aspects of PHA. It is clear that at the end of the 3rd millennium, transgenic PHA producers will be an important source of green plastics and chemicals to the world. With the advent of further developments in metabolic engineering, such biotechnologies will be the rule rather than an exception. To discuss in great detail the vast number of microorganisms capable of producing PHAs would be beyond the scope of this review.

With all these advances it is likely that PHAs will become a major biodegradable plastic in a wide range of applications in the near future and will eliminate the disposable problem and environmental hazard, as are prevalent with conventional plastic material.

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