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Key Insights of Natural Bioplastic Polyhyroxybutyrate (PHB) Synthesis In Cyanobacteria

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ABSTRACT

The limited fossil fuel and nonbiodegradability of conventional petrochemical based plastics prompted scientist for search of environmental friendly alternatives. Bioplastic produced from renewable sources appeared as a better alternative. The bioplastics available in the market are made from polymers such as starch-based, polyhydroxyalkanoates (PHAs), polylactic acid (PLA) etc. But attributed by its numerous advantages including those of completely biodegradable, PHAs are considered to be the most desired one. Cyanobacteria are highly promising microorganism for PHA production. This review highlights the basic biology of cyanobacterial PHA production, strains involved, recombinant PHA production and its future prospects. While integrating the existing knowledge and technology, future improvement and progress are to be done before commercial exploitation of cyanobacterial PHA production.

Keywords: Biodegradable plastics; Cyanobacteria; Petroleum based plastics; PHA synthases; Polyhydroxyalkanoates (PHA); Polyhydroxybutyrate.

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INTRODUCTION

Plastics have become an indispensable commodity to improve the comfort and quality of life. Since 1940s plastics have been replacing glass, wood, metal and other constructional materials in numerous applications[1]. Plastic has multipurpose qualities of strength, lightlessness, stability and resistance to degradation. The nonrenewable sources of petrochemicals as petroleum are predicted to end in near future. Moreover, their very desirable properties have now become the greatest problem. Accumulation of recalcitrant plastic in the environment is a world-wide problem as globally about 30 million tones plastic waste accumulates in the environment per year[2]. Solutions for the plastic management include, incineration, recycling and photo-degradation. However, most of these management procedures lead to serious environmental pollution[3]. Not only this, the manufacturing processes required to produce plastic also create large quantities of chemical pollutants. Combustion of acrylonitrile-based plastics release toxic hydrogen cyanide that causes serious health hazards[4]. Besides this, a large amount of green house gases are emitted that are major threat of this century. Presence of pigments, coatings materials, filling agents limits the use of the recycled materials. Their disposal in the landfills results in leaching of toxic chemicals that severely affects the adjacent ecosystem. Besides this, aquatic fauna die by ingesting plastic or by getting entangled in them.

A completely biodegradable polymer is identified as a polymer that is entirely transformed to carbon dioxide, water and humic material by living organisms, usually microorganisms present in soil, sea, lake water and sewage[5]. Biodegradable polymers under development include polyglycolic acids, aliphatic polyesters, polylactides, polyhydroxyalkanoates (PHAs), polysaccharides and their co-polymers and/or blends[6]. Amongst these, PHAs are of special interest because of their likeness to synthetic polymers to a larger extent and their thermoplastic nature required for processing[7]. A number of bacteria such as Archaebacteria, *Azotobacter*, *Bacillus*, Methylobacteria, *Pseudomonas* synthesize PHA to varying levels ranging from 50% to 80% of the dry cell mass[8]. In order to mass-produce PHAs, various wild types as *Ralstonia eutropha*, *Alcaligenes latus* and recombinant *E. coli* strains have been studied[9]. High investment for exogenous carbon source, small production volumes, and expensive PHA recovery processes, particularly for purification are the major obstacles faced for its commercial production[10]. Bacterial PHA is 5–10 times more expensive than the petroleum-derived polymers.

PHA synthesizing genes from wild bacteria like *R.eutropha*, *A.latus* were transferred into plant system for developing photosynthetic PHA production system[11]. Very low PHA content (generally less than 1 % dry weight), long life span with stunted growth, fertility problems and difficulties in isolation of PHA from cellular components is the limitation of PHA production through genetically engineered plants[12]. Hence PHA producing cyanobacteria with added advantage of shorter generation time and simple inorganic nutrients requirement attracted the researchers. Cyanobacteria are known to accumulate PHB --- the most studied and characterized PHA. But there are reports of accumulation of copolymers like poly(3-hydroxybutyrate-co-valerate) (PHBV)[13].

BIOSYNTHESIS OF PHB

Biosynthesis of PHB represents the simplest PHA biosynthetic pathway[14]. It consists of three enzymatic steps (Figure 1). In the first step, two molecules of acetyl CoA condense to form acetoacetyl CoA, the reaction being catalysed by β-ketothiolase (acetoacetyl- CoAthiolase; EC 2.3.1.9). In the second step, acetoacetyl CoA is reduced to (R)-3-hydroxybutyryl-CoA in the presence of an NADPH dependent acetoacetyl-CoA reductase (EC 1.1.1.36). In the last step, the (R)-3-hydroxybutyryl-CoA is polymerized to PHB by a PHA synthase (E.C. 2.3.1.-).

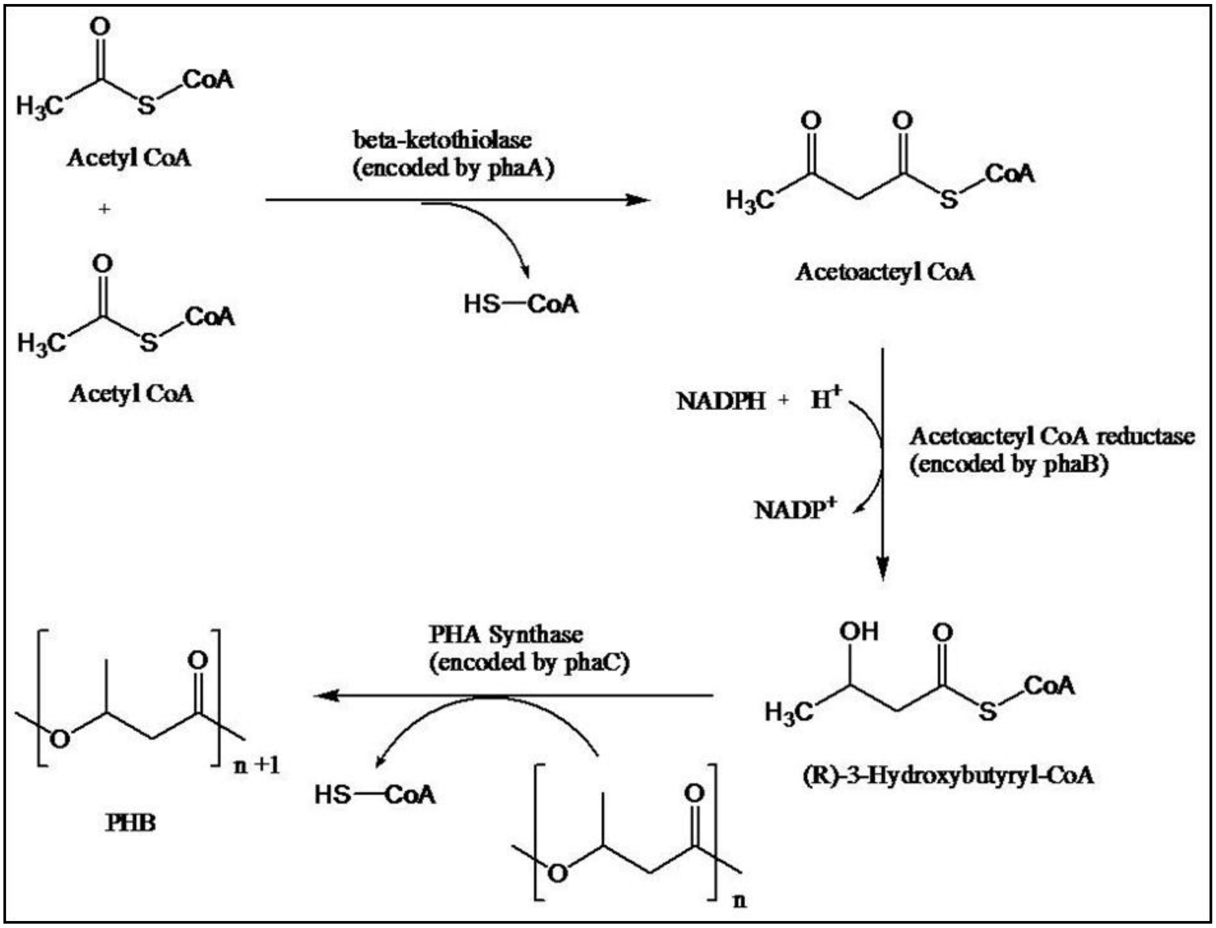

Figure 1: Biosynthetic pathway of PHB

PHA SYNTHASE

PHA synthase is the key enzymes for PHA biosynthesis and is called as the natural catalyst for plastics synthesis[15]. Based on the primary structures (subunit composition) and substrate specificity, PHA synthase is classified into four classes (Figure 2).

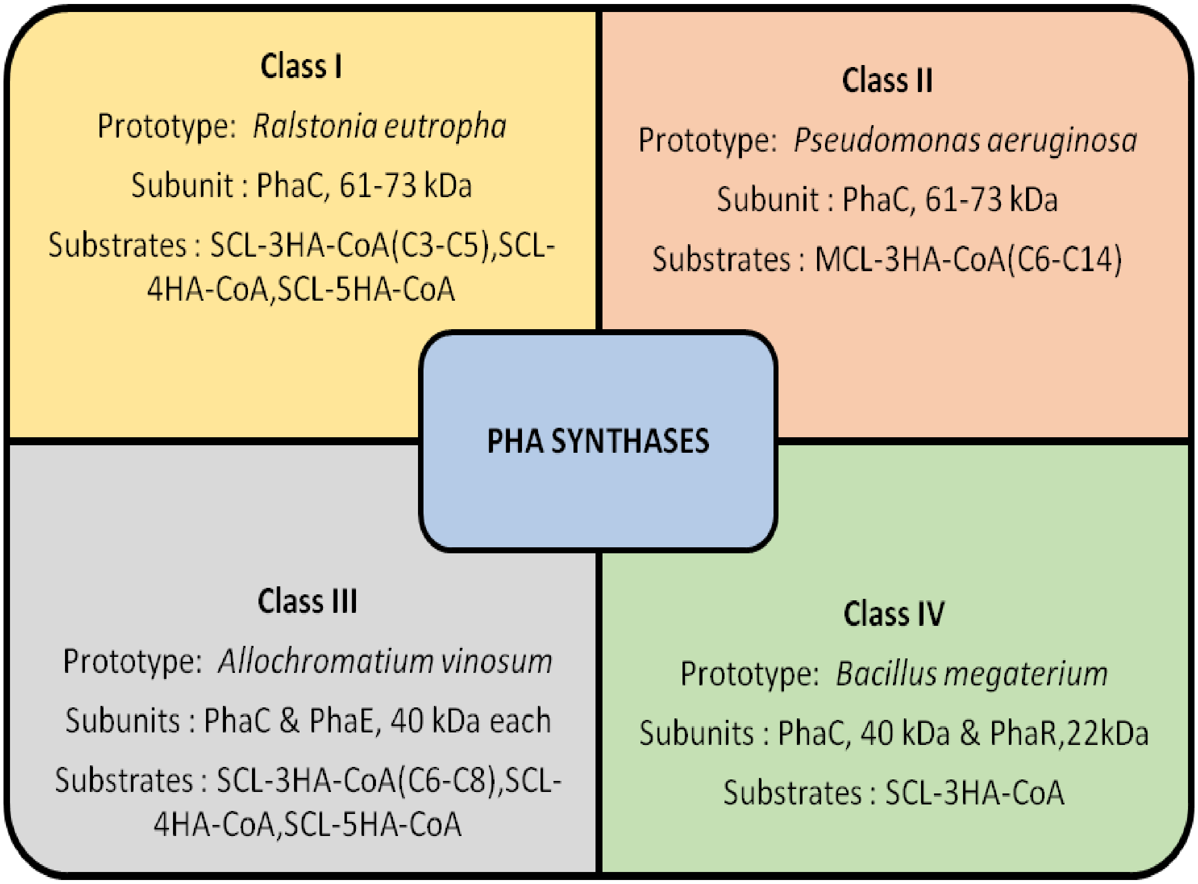

Figure 2: Types of PHA synthases based on subunit composition and substrate specificity

Class I and class II PHA synthases consists of only one type of subunit (PhaC) with 61-73 kDa molecular masses but *in vivo* and *in vitro*experiments revealed dissimilarity in their substrate specificity[16]. Class I PHA synthases has a wide substrate specificity as besides preferentially using short chain length (SCL) 3-hydroxyfatty acids (C3-C5), it utilises 4-hydroxyfatty acids and 5-hydroxyfatty acids whereas class II has a narrow substrate specificity utilising medium chain length (MCL) 3-hydroxyfatty acids (C6-C14) only[17].

In addition to PhaC, type-III PHA synthases has subunit PhaE (40 kDa each). Type III PhaC subunit exhibits only 21 to 28% amino acid sequence similarity to type I and II PhaC and possses only negligible PHA synthase activity. It is dependent on PhaE subunit for its activity[18,19]. Two C-terminal amino acid stretches of PhaE proteins helps in binding the Type III synthase to the PHA[20]. The substrate specificities of Type III synthases are not very well defined, but generally they prefer short chain length (SCL) 3-hydroxyfatty acids (C6-C8)[21]. Class IV PHA synthases consists of two subunits like class III PHA synthases, but here PhaE is replaced by 22 kDa PhaR[22]. Short chain length (SCL) 3-hydroxyfatty acids (C3-C5) are the preferred substrates for this group of synthases. Though the tertiary structure of PHA synthases has not yet been determined but the multiple alignment has revealed that they are mixed class of proteins consisting of α-helix, random coil and β-sheet[15]. Protein BLAST analysis of the PHA synthases sequences showed convincingly high homology to different lipases (from bacteria, fungi or mammals)[15]. These lipases are serine esterases belonging to the α/β hydrolase superfamily, in which nucleophilic serine, part of a conserved Ser-His-(Asp/Glu) triad, is located in an extremely sharp turn termed nucleophilic elbow[23]. However, PHA synthases contain cysteine residue in place of serine of the lipases as the active site nucelophile. Threading models for all four classes of PHA synthases has been proposed using the crystallographically resolved protein structure of lipases (Figure 3a-3d). For generating Class I PHA synthase model (PhaC of *R. eutropha*), crystallographically resolved protein structure of *Burkholderia glumae* lipase was used. The resulting protein model consist of active site Cys-319, the conserved His-508 and the Asp-480 forming the catalytic triad (Figure 3a) with active site Cys-319 located at the nucleophile elbow. An analogous approach was conducted to build a structural model of the class II PHA synthase (PhaC of *P. aeruginosa*) using the crystallographically resolved protein structure of epoxide hydrolase from mouse[17]. Its catalytic triad consists of active site Cys-296, the conserved His-480 and the Asp-452with active site Cys-296 residing at the nucleophile elbow (Figure 3b). For proposing threading model for class III PHA synthase (PhaC of *A. vinosum*), the corresponding α/β - hydrolase domain of *Burkholderia cepacia* lipase was analyzed (Figure 3c)[19]. The resulting model revealed that the conserved residues His-331, Asp-302 and His-303 form the triad. Previously Cys-130 was thought to exist at the conserved nucleophile site[24] but later it was reported that Cys-149 is located at the active nucleophile site.[19] Similarly to the above three synthases, Class IV PHA synthase from *B. cereus* SPV was found to bear sequence-homology to human gastric lipase[23]. The threading model comprises the active site Cys-151, the conserved His-335 and the Asp-306 together forming the catalytic triad (Figure 3d).

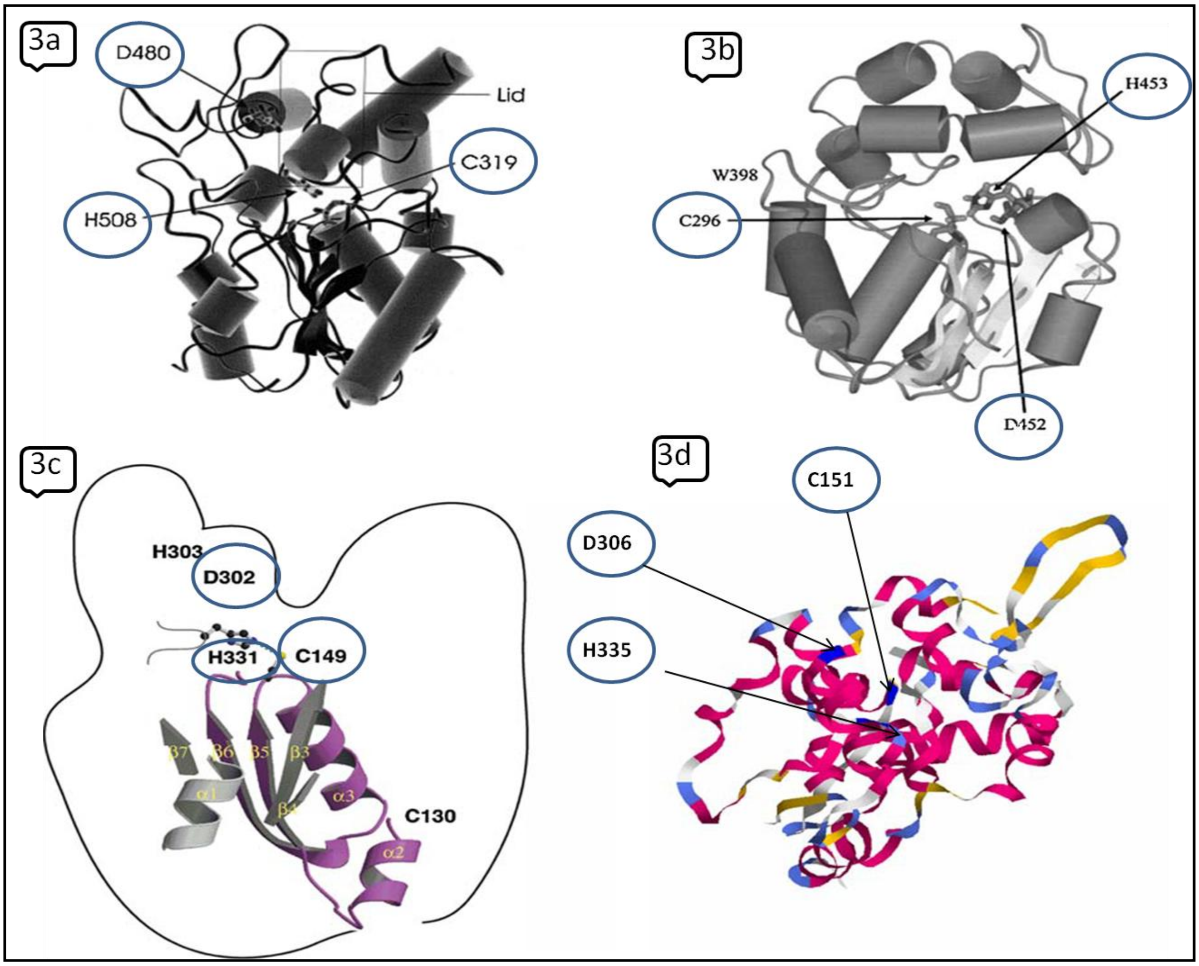

Figure 3: Schematic models for PHA synthases. (a) Class I PhaC of *R. eutropha*, (b) Class II PhaC of *P. aeruginosa*, (c) Class III PhaC of *A. vinosum* and (d) Class IV PhaC of *B. cereus* SPV. Catalytic triad residues (cysteine–aspartate– histidine) are encircled [25]

CYANOBACTERIAL PHA SYNTHASE

Though accumulation of polyhydroxyalkanaotes in cyanobacteria has attracted the attention since the report of PHB production in N_2-fixing cyanobacterium, *Chlorogloea fritschii* but identification, cloning, and molecular characterization of cyanobacterial PHA synthase could be reported for the first time in the unicellular cyanobacterium *Synechocystis* sp. PCC6803[26,27]. They revealed that PHA synthase of *Synechocystis* sp. PCC6803 is a two-component enzyme and is represented by two open reading frames (ORFs) viz. slr1830 and slr1829. The ORF slr1830 encoding a protein with 378 amino acids was designated as *phaC* ($phaC_{\mathrm{Syn}}$) whereas the other ORF slr1829, located colinear and upstream of *phaC* was designated to as *phaE* ($phaE_{\mathrm{Syn}}$). Multialignment of the *phaE* and *phaC* gene products exhibited striking sequence similarities to the corresponding class III PHA synthase subunits PhaE and PhaC of three γ-*Proteobacteria* viz. *Chromatium vinosum, Thiocystis violacea and Thiococcuspfennigii* (Figure 4).

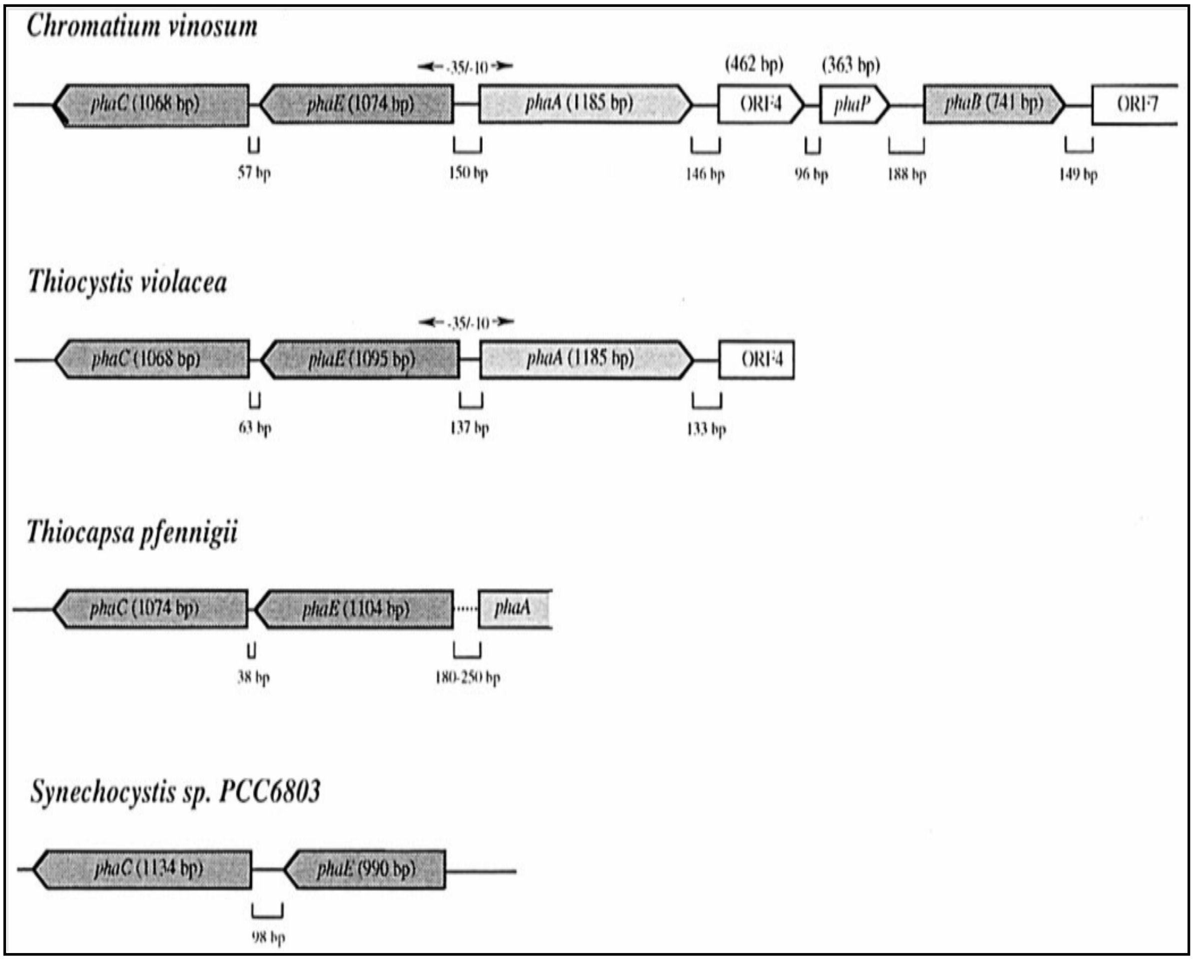

Figure 4: Occurence of type III PHA synthase characteristic of γ-*Proteobacteria* in *Synechocystis* sp. PCC6803[21]

They confirmed these molecular data conclusions through heterologous expression of *phaE*$_{\text{Syn}}$, and *phaC*$_{\text{Syn}}$ genes, along with the *Alcaligenes eutrophus* genes *phaA*$_{\text{Ae}}$ and *phaB*$_{\text{Ae}}$ encoding the β-ketothiolase and acetoacetyl CoA reductase, respectively in *Escherichia coli* S17-1 and also through phenotypic complementation of a PHA-negative mutant of *Alcaligenes eutrophus*. In the subsequent studies, a more confirmative and conclusive proof for the requirement of co-expression of two genes, *phaC* and *phaE* for a functionally active PHA synthase in *Synechocystis* sp. PCC6803 was shown by constructing a PHA negative *Synechocystis* sp. strain through precise disruption of the phaE$_{\text{Syn}}$ by long flanking homology polymerase chain reaction (LFH-PCR)[28]. The phaEnegative mutant strain showed no accumulation of PHB even under high PHB accumulating conditions in contrast to the wild type strain. Later studies provided multiple evidences for widespread and general occurrence of type-III PHA synthases in cyanobacteria[29]. Several methods, including (i) Southern blot investigation using a precise *phaC* DNA probe, (ii) Western blot study using specific polyclonal anti-PhaE antibodies, (iii) PCR products generation using *phaC* specific primers and their sequence analysis, and/or (iv) cloning and sequence analysis of PHA synthase structural genes, were used to provide evidence for the presence of a type-III PHA synthase in the eleven cyanobacteria[29]. They successfully cloned, sequenced and heterologously expressed the entire PHA synthase genes of thermophilic cyanobacteria *Synechococcus* sp. strain MA19 and *Chlorogloeopsis fritschii* PCC 6912, and the central region of *phaC* gene of *Cyanothece* sp. strain PCC 8303in *Escherichia coli*. Further, the amino acid sequences of PhaC of the above three cyanobacteria exhibited highly conserved regions adjacent to the positions corresponding to Cys-149, Asp-302, His-303 and His-331 residues in the sequence of *Allochromatium vinosum* which serve as the covalent catalysis site similar to the active centres of class III PHA synthases.

β-KETOTHIOLASE AND ACETOACETYL-CoA REDUCTASE

Identification and characterization of the other two PHA synthesizing genes coding for the enzymes β-ketothiolase (*phaA*) and an acetoacetyl-CoA reductase (*phaB*) respectively was also done in *Synechocystis* sp. strain PCC6803[30]. They identified a cluster of two putative open reading frames, slr1993 and slr1994 encoding 409 and 240 amino acids long proteins for these two genes ($phaA_{Syn}$and $phaB_{Syn}$) respectively. PHA accumulation (up to 12.3% of the cell dry weight) under high-glucose growth conditions occurred with heterologous expression of the two genes ($phaA_{Syn}$and $phaB_{Syn}$) together with the PHA synthase genes of *Synechocystis*, $phaE_{Syn}$and $phaC_{Syn}$, in *E. coli* whereas targeted disruption of the $phaA_{Syn}$and $phaB_{Syn}$genes through long flanking homology-PCR resulted in the loss of PHA-producing capacity in the organism. They also revealed that the *Synechocystis* PHA-specific thiolase and reductase has high degree of homology to those found in representatives of the γ-*Proteobacteria*. Though the PHA biosynthesis genes sequences of *Synechocystis* showed much similiarity to that found in these three γ-proteobacteria but their organization in *Synechocystis* was different. In the case of *Synechocystis* PHA biosynthesis genes form two separate clusters, $phaA_{Syn}$and $phaB_{Syn}$ cluster located between 1.43 to 1.44 mnt and $phaE_{Syn}$and $phaC_{Syn}$located between 0.932 to 0.934 mnt (Figure 5) whereas biosynthetic genes, *phaA* and *phaB* are contiguous with the *phaE* and *phaC* genes in the above three γ-proteobacteria[21].

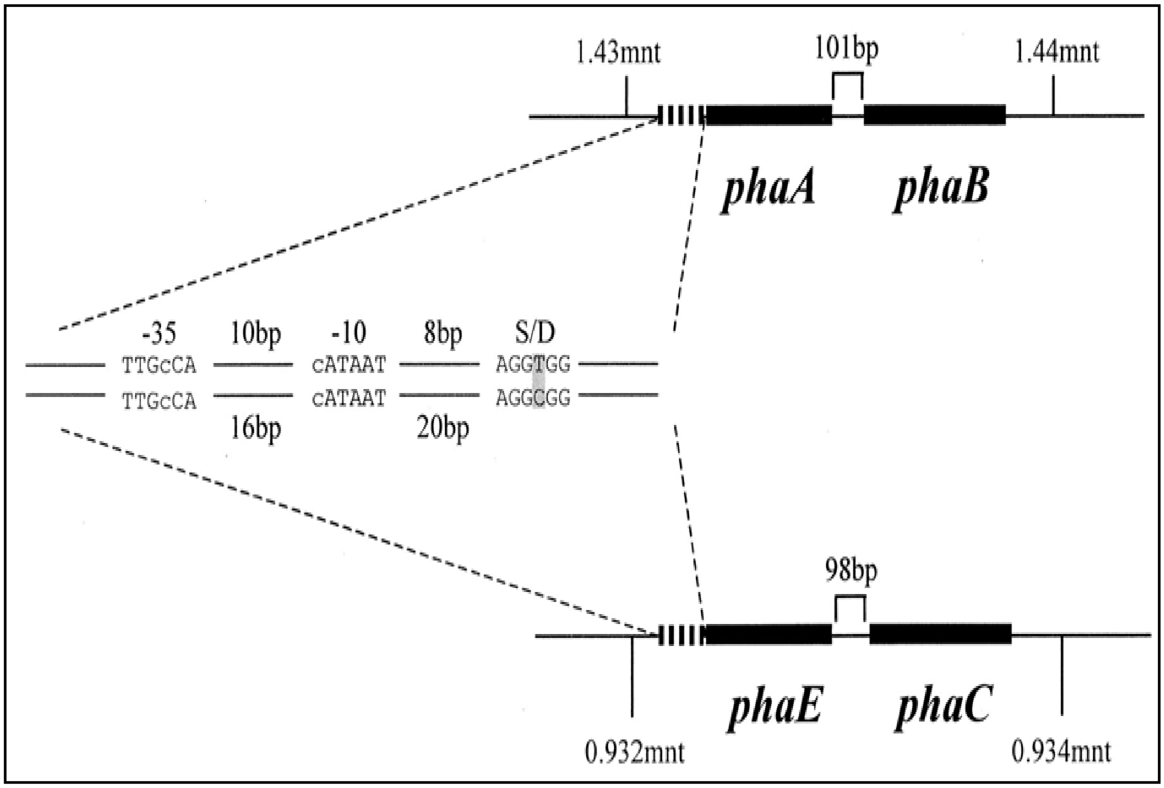

Figure 5: Organization of the PHB biosyntheticgenes in *Synechocystis* sp. strain PCC6803[30]

PHB ACCUMULATING CYANOBACTERIA

Since the discovery of PHB in the cyanobacterium, *Chloroglea fritschii* as stated above, the occurrence of PHB has been shown in several other species (Table 1).

Generally low PHB content (<10% dcw) has been reported in cyanobacteria under photoautotrophy[32,35]. But researchers had tried to increase the PHB accumulation in cyanobacteria by altering the culture conditions and or using genetic engineering.

Table 1: PHB synthesizing cyanobacteria[31-34]

Anabaena cylindrica	*Anabaena variabilis*	*Anabaena doliolum*
Anacystis cyanea	*Anabaena hallensis*	*Aphanocapsa sp*
Aulosiara fertilissima	*Calothrix brevissima*	*Chroococcus sp*
Chloroglea fritschii	*Cyanothece*	*Cyanospira rippkae*
Fischerella muscicola	*Gloeobacter violaceus*	*Gloeocapsa sp*
Gloeocapsa gelatinosa	*Gloeotrichia raciborskii*	*Hapalosiphon fontinalis*
Lyngbya aestuarii	*Lyngbya sp*	*Mastigocladus laminosus*
Michrochaete sp	*Microcoleus sp*	*Microcystis aeroginosa*
Nodularia sp	*Nodularia haveyana*	*Nostoc sp*
Nostoc commune	*Nostoc linckia*	*Nostoc muscorum*
Nostoc paludosum	*Nostoc punctiforme*	*Oscillatoria limnetica*
Oscillatoria limosa	*Oscillatoria sp*	*Phormidium borianum*
Phormidium sp	*Pleurocapsa sp*	*Rivularia sp*
Scytonema sp	*Spirulina jenneri*	*Spirulina laxissima*
Spirulina maxima	*Spirulina platensis*	*Spirulina subsalsa*

Synechococcus MA19	*Synechocystis PCC6803*	*Synechocystis sp*
Tolypothrix tenuis	*Trichodesmium thiebautii*	*Westiellopsis prolifica*

PHB PRODUCTION UNDER ALTERED CULTURE CONDITIONS

Altered culture conditions employed for PHB production are generally exogenous supplementation of carbon sources like glucose, acetate etc. and or deficiency of nitrogen or phosphorus[36]. Acetyl-CoA is the precursor molecule needed for PHB synthesis[14]. Excessive acetyl CoA left, besides being used for TCA cycle, fatty acyl-ACP synthesis (for cell growth) is needed for PHB synthesis in cyanobacteria[14,37]. Acetyl-CoA can be enhanced by exogenous supplementation of acetate and also other carbon sources which are catabolised into acetyl CoA inside the cyanobacterial cell. In cyanobacteria, exogenously supplied carbon souces catabolize through oxidative pentose phosphate pathway that increases NADPH pool[38]. It is the cofactor of acetoacetyl-CoA reductase, the second enzyme of PHB biosynthetic pathway[39]. Phosphorus deficiency also increases the NADPH pool as phosphorus deficiency causes decrease in ATP production whereas the reduction of NADP yielding NADPH through noncyclic photosynthetic electron flow continues resulting in increased NADPH pool[40]. *H*eterotrophic growth of cyanobacteria is dependent mainly on the dark period. Hence dark conditions are often applied with exogenous carbon supplementation. Nitrogen deficiency causes induction of *sll0783* gene, the product of which is required for the third enzyme PHB synthase activity post-transcriptionally in *Synechocystis sp*. strain PCC 6803 leading to an enhanced PHB synthesis[41].

PHAs are known to have dual function as a reserve compound and also as a stress metabolite accumulating in response to stress condition. Therefore, stressed conditions as dark, anoxic, salt-stressed or nutrient-starved growth conditions lead to an increase in PHB content. A direct effect of NaCl concentration on citrate synthase in the TCA cycle results in an increased acetyl CoA pool that favours PHB synthesis. It was also suggested that by storing large quantities of reduced carbon in the form of PHB during osmotic stress, salt tolerant bacterial cells might balance the osmotic pressure imposed by the environment[42]. The effect of above culture conditions resulting in increased PHB production can be summarized as in Figure 6 and Table 2.

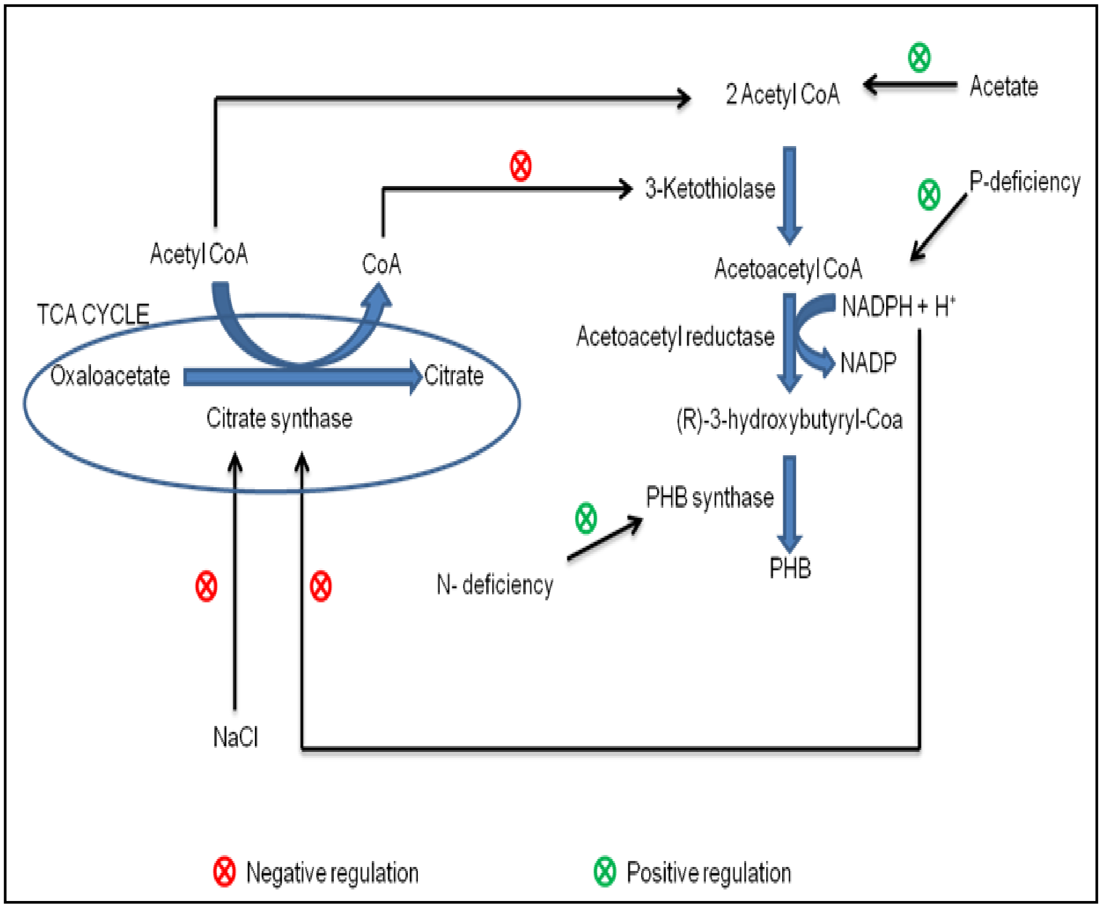

Figure 6: Interactions of physicochemical conditions with PHB biosynthesis

Table 2: Cyanobacterial PHA production under altered culture conditions

Cyanobacteria	Carbon supplement	Limiting parameter	PHA (%)	Reference
Chloroglea fritschii	Acetate	---	PHB(10%)	Ref[26]
Spirulina maxima	Acetate	---	PHB(3.1%)	Ref[43]
Spirulina platensis	Acetate	---	PHB(2.9%)	Ref[44]
Oscillatoria limosa	Acetate	---	PHBV(6%)	Ref[31]
Anabaena cylindrica 10C	Acetate and Propionate	---	PHBV(2%)	Ref[45]
Synechococcus MA19	CO_2	N_2 starvation and dark	PHB (27%)	Ref[46]
Spirulina platensis UMACC 159,161,162	Acetate	N_2 starvation and dark	PHB (10%)	Ref[47]
Synechocystis PCC6803	CO_2	N_2 starvation	PHB(4.1%)	Ref[33]
	Acetate	N_2 starvation	PHB(15.2%)	Ref[48]
Synechococcus MA19	CO_2	Phosphate deficiency	PHB (55%)	Ref[49]
Synechocystis PCC6803	Acetate	---	PHB (7%)	Ref[50]
Nostoc muscorum	Acetate	Dark	PHB (43%)	Ref[36]
Synechocystis PCC6803	Acetate and Glucose	Phosphate deficiency	PHB (29%)	Ref[51]
	Acetate	Dark	PHB (22%)	---do---
	Acetate and Fructose	Phosphate deficiency and Gas exchange limitation	PHB (38%)	---do---
Synechocystis sp UNIWG	Acetate	N_2 starvation	PHB (14%)	Ref[52]
Spirulina sp UMACC	---do---	---do---	PHB (10%)	---do---
Aulosira fertilissima	Acetate and Citrate	Phosphate deficiency	PHB (85%)	Ref[53]
Nostoc muscorum Agardh	Acetate and Valerate	N_2 starvation	PHBV(60%)	Ref[54]
Aulosira fertilissima	Fructose and Valerate	---	PHBV(77%)	Ref[13]

GENETIC ENGINEERING FOR PHB PRODUCTION

For the first time, transformation of PHB synthesizing genes from bacteria *Alcaligenes eutrophus* into non PHB accumulator *Synechococcus sp.* PCC7942 was studied in last decade of 20th century[55]. It accumulated 1% PHB under photoautotrophic nitrogen deprived conditions. Subsequent studies used an improved vector with a strong promoter for a slight increase in PHB content in nitrogen starved conditions (upto 3%) but drastic increase was reported (upto 25%) on acetate supplementation[56]. Parallely, transformation of marine cyanobacterium *Synechococcus sp.* PCC7002 with the PHB genes from *R. eutropha* was studied to report accumulation of copolymer of hydroxybutyrate and hydroxyvalerate[57]. *Synechocystis sp* MA 19, a potent thermophilic PHB accumulator was genetically engineered by inserting the transposon Tn5 from *E.coli* S17-1[58]. The mutant showed higher PHB accumulation rate than the wild under nitrate deprivation. Later enhancement in PHA synthase activity was made by transformation of *Ralstonia eutropha* PHA biosynthetic operon in wild type *Synechocystis* sp PCC6803 with *E. coli* S17-1 having plasmid vector containing PHA genes using the spot-mating technique. But only a marginal increase in PHA content was noticed in the recombinant even after subjected to nitrogen starved conditions with different carbon sources[50]. Large scale PHA production from recombinant cyanobacteria using costly antibiotic is not economical as recombinant cyanobacterial PHB production through plasmid mediated tranformation needs antibiotic for plasmid maintainance. Upto 52% antibiotic free PHA is reported in recombinant *Synechococcus* PCC7002 under nitrogen starvation[59]. More recently, β-ketothiolase of *Synechocystis sp.* PCC 6803 has been substituted by an acetoacetyl-CoA synthase (nphT7Ss) from *Streptomyces sp.* CL190 that catalyzes the irreversible condensation of acetyl-CoA and malonyl CoA to give acetoacetyl-CoA, resulting in as 14 wt% PHB under photoautotrophic conditions without the addition of a carbon source[60]. However under air exchange limiting conditions with acetate supplementation, a significant increase (up to 41 %) in PHB was observed.

CONCLUSIONS

This study shows that cyanobacteria can be a good source for biodegradable plastic. Though cyanobacteria produce predominantly PHB but are also competent to synthesize copolymers like PHBV under proper substrate feeding. Alternations in culture conditions and or genetic engineering techniques can be used for increasing PHB production level in cyanobacteria. Also PHB production via cyanobacteria offers a potential eco-friendly way as it utilises the ‘green-house gas’ into biodegradable plastics photosynthetically. Therefore, exploitation of cyanobacteria for PHB production with carbon dioxide and sunlight seems highly promising as it has the advantage of producing polymer without expensive carbon source and oxygen supplementation.

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