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Review article

A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics

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ABSTRACT

The increasing effect of non-degradable plastic wastes is a growing concern. As an alternative, researches are being attempted from living resource to produce bio plastics on the basis of their biodegradability. Due to their cost effect nature, now the scientists are searching an alternative resource like photoautotrophic cyanobacteria. In this review the promising importance and growing awareness of using cyanobacteria as PHB resource are being reported. Many publications evidenced that various cyanobacterial species accumulate intracellular poly-β-hydroxybutyrate granules as energy and carbon reserves inside their cells when they are in stress conditions. PHB is biodegradable, environmental friendly and biocompatible thermoplastics. Varying in toughness and flexibility, depending on their formulation, they can be used in various ways similar to many non-biodegradable petrochemical plastics currently in use. Promising strategies involve genetic engineering of microorganisms to introduce production pathways are being investigated for the past two decades. Such kind of researches focusing on the use of alternative substrates, novel extraction methods, genetically enhanced species and mixed cultures with a view to make PHB from cyanobacteria (blue green algae) more commercially attractive are presented and discussed.

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1. Introduction

Poly-β-hydroxybutyrate is a wide spread intracellular storage compound typically in prokaryotic organisms [1–5]. The properties of pure poly-β-hydroxybutyrate including thermoplastic process ability, absolute resistance to water and complete biodegradability suggest that PHB could be an attractive to common plastics and would fit well with new waste management strategies [6–9]. The use of PHB produced by bacterial fermentation as a commodity polymer is limited by its high production cost compared to some widely used petroleum derived plastics. The number as well as the types and potential qualities have greatly increased the production of superior materials such as epoxides, and polysulfones, and have become one of the most widely used products all over the globe [10–14].

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Durability and resistance to degradation are desirable properties when plastics are in use, but they pose problems for disposal when out of use. These non-biodegradable plastics accumulate in the global environment at a rate of 25×10^6 t per year passing serious threats to the solid waste management program [15–18]. In today's modern era of science and technology plastics have become one of the most widely used materials all over the world [19–23], and applications are nearly universally important in automobiles, home appliances, computer equipment packages and even medical applications. The quality of plastics and its uses in day today life have long been vilified because they are environmentally unfriendly and they are not biodegradable [24–27]. So today's demand for biodegradable plastics is one of the most important targets both for basic and applied research.

In the early 1920s, Lemoigne a microbiologist at Pasteur Institute in Paris isolated a polymer from *Bacillus megaterium* by chloroform extraction and demonstrated that it was a polyester of 3-hydroxybutyric acid [28–32]. Since Lemoigne discovered PHB, the polymer has presented many challenges to microbiologists and biochemists who are interested in its physiological functions and metabolism. The general knowledge of microbial PHB was first summarized in a comprehensive review by Dawes and Senior in the year 1973. Later, it was found that PHB is only one type in a huge family of polymers collectively known as polyhydroxyalkanoate (PHA). In 1974, PHB was isolated by chloroform extraction of activated sludge [33–35]. The monomers that were detected in chloroform extracts of activated sewage sludge are 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HH) as the major and minor constituents respectively. About a decade later following the identification of heteropolymers, the analysis of marine sediments by capillary gas chromatography revealed the presence of 3HB and 3HV as the predominant components among 11 other short chain 3-hydroxyalkanoate monomers [36–38]. Likewise, research on finding new PHBs is also in the streamline.

Among the 150 different types of polyhydroxyalkanoids identified so far, the homopolymer of hydroxybutyrate like PHB is widespread in different taxonomic group of prokaryotes including cyanobacteria. The properties of pure PHB including thermoplastic processibility, hydrophobicity, complete biodegradability and biocompatibility with optical purity have increasingly become of interest as a raw material for biodegradable plastics [15,39,40]. Cyanobacteria can be considered as an alternative host system due to their minimal nutrient requirements and photoautotrophic nature. Cyanobacterial species have the ability to accumulate the homopolymer of PHB under photoautotrophic condition [41,42,26] Cyanobacteria are capable of accumulating PHB. Industrial utilization of cyanobacteria as PHB producers has the advantage of converting waste carbon dioxide, a greenhouse gas to environmental friendly plastics using the energy of sunlight. Various species of cyanobacteria accumulate considerable amounts of PHB [43,44]. Based on the literature available on the cyanobacterial PHB production, this review has been compiled and reported with a clear view on the current status, future prospect and needed improvement in this area.

2. Chemical composition and physical properties of poly-β-hydroxybutyrates

Poly-β-hydroxybutyrate is synthesized from acetyl coenzyme A (acetylcoA) via three enzymatic reactions. 3-Ketothiolase converts two acetyl-coA molecules to one acetoacetyl-coA molecule, NADPH dependent acetoacetyl–coAreductase converts acetoacetylcoA to D-3-hydroxybutyrylcoA and the last enzyme PHB synthase catalyzes linking of the D-3-hydroxybutyryl moiety to an existing PHB molecule via an ester bond shown in the Fig. 1. However, β and many other PHA are composed of 3 hydroxy fatty acids.

The pendant group varies from methyl carbon 1 to tridecyl carbon 13. Fatty acids with the hydroxyl group at position 4, 5 and 6 pendant group containing substituents or unsaturation's are known. PHB and PHV (poly 3 hydroxyvalerate) form a class of PHA and are typically referred to as short chain length PHAs. In contract medium the chain length of PHAs is composed of carbon 6 to carbon 16 3-hydroxy fatty acids [45,46]. It has been suggested that PHB homopolymer synthesized by bacteria always contains less than 1 molecule of 3% hydroxyvalerate monomers [34]. The most common polymers are shown in Table 1.

Copolymers of PHB are formed when mixed substrates are used such as a mix of glucose and valerate. The microorganisms convert the substrates into small chain linked PHAs like poly (3 hydroxybutyrate co 3 hydroxyvalerate) PHBV or poly (3hydroxybutyrateco 4 hydroxybutyrate) PHB4B [44,47]. PHBHx copolymers that contain 3-hydroxyl hexanoate units and other molecule PHAs are reported [29]. When a mixture of substrates is used the resulting polymers are random copolymers. When the substrates are alternated overtime, it is possible to obtain PHA block copolymers synthesized by bacteria. The chemical structures are shown in Fig. 2.

Bacteria produce PHBs with average molecular mass of up to 4.6×10^6 Da with a polydispersity (Mw/Mn) of around 2.0 [48]. The material characteristics of these biopolymers are similar to conventional plastics such as polypropylene [30,34,49]. The properties of PHB (homopolymer), PHBV, PHB4B (scl-copolymers) and PHBHx (mcl-copolymer) are compared with polypropylene (PP) in Table 2. PHB homopolymer is a highly crystalline [32], stiff, but brittle material. When spun into fibers it behaves as a hard elastic material [37]. Copolymers like PHBV or mcl-PHAs are less stiff and brittle than PHB, while retaining most of the other mechanical properties of PHB. Homopolymer PHB has a helical crystalline structure, this structure seems to be similar in various copolymers [38]. Melting behavior and crystallization of PHB have recently been studied [50,51]. The physical properties have been differentiated in Table 2.

3. Poly-β-hydroxybutyrate production in cyanobacteria

Cyanobacteria can be considered as an alternative host system due to their minimal nutrient requirements and photoautotrophic nature for the production of PHB. Cyanobacterial species have the ability to accumulate the homopolymer of PHB under photoautotrophic condition. Cyanobacteria a group of oxygen evolving photosynthetic bacteria with a short generation time need some simple inorganic nutrients such as phosphate, nitrate, magnesium and calcium as macro and ferrous, manganese, zinc, cobalt, and copper as micronutrients for their growth and multiplication [52–55]. The first cyanobacterial species which reported the presence of PHB is *Chlorogloeafritschii* in the year 1966. To date, the occurrence of PHB has been demonstrated for several cyanobacteria such as *Spirulina* spp., *Aphanothece* spp., *Gloeothece* spp., and *Synechococcus* spp. [56–62]. The *Synechocystis* spp. possesses genes corresponding to PHB synthase and conducted research on its characteristics [63,64]. The detection of PHB was done electron microscopically respectively in *Gleocapsa* spp. and *Nostoc* spp. under photoautotrophic conditions [65–68]. Oval structures resembling PHB granules were also detected by means of ultra-structural analysis in *Microcystis aeruginosa* and *Trichodesmium thiebautti*. The presence of PHB in *Oscillatorialimosa* spp. and *Gloeothece* spp. is detected by Gas–liquid chromatography [69–71]. These organisms can successfully be cultivated in waste waters due to their ability to use inorganic nitrogen, phosphorous and wastewaters such as effluents of farmyards, fish farms, rubber industries, and sewage treatment plants, which are rich sources of nitrogen and phosphorous [72–74]. The relatively low weight of PHB in the cyanobacterium when compared to other bacteria was probably due to the small size and mass. They also reported that PHB synthesizing ability of the cyanobacterium might, in fact, be quite similar to that was shown by most bacteria in nature. The advantage of the environmental friendly and highly processes synthesis of biodegradable and in many cases biocompatible polymers will become increasingly attractive for the industry.

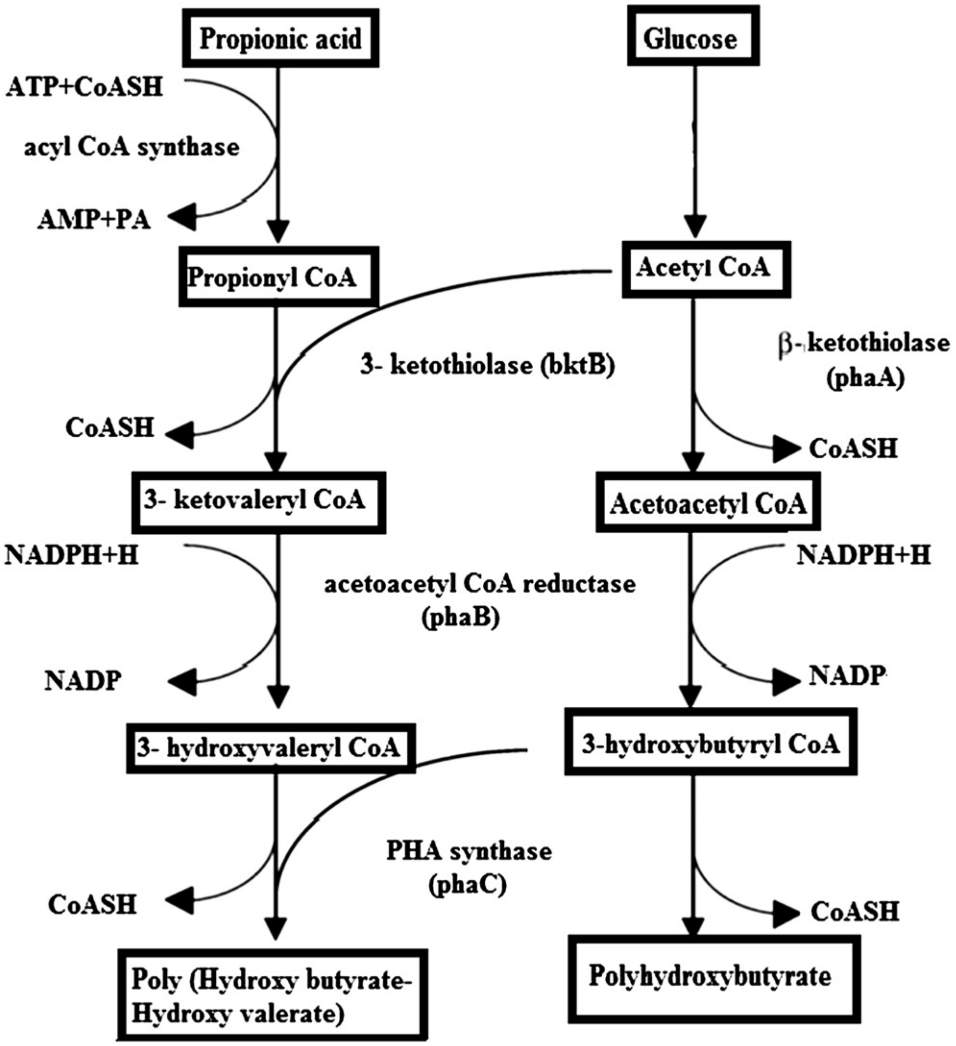

Fig. 1. Poly-β-hydroxybutyrate production pathway.

4. Cyanobacteria and poly-β-hydroxybutyrate

4.1. Cyanobacterial systems and their capability of producing PHB

Cyanobacteria have a mechanism for storing phosphate predominantly in the form of polyphosphate and during phosphate deficiency these organisms counter the paucity of π by breaking the polyphosphate chains [75–77]. Industrial utilization of cyanobacteria as PHB producers has the advantage of converting waste carbon dioxide, a greenhouse gas to environmental friendly plastics using the energy of sunlight. Various species of cyanobacteria accumulate considerable amounts of PHB. Non PHB accumulating cyanobacteria have recently been genetically engineered by transformation with genes involved in the PHB pathway [78,79].

The dormant cells of cyanobacteria are the most widespread and well-studied cell type responsible for the persistence of the populations. Akinetes are differentiated cells with a number of specific ultra-structural characteristics, including the thickened peptidoglycan layer of the cell wall, an additional multilayered envelope and high content of storage polymers [80,81]. Akinete formation results from a limitation of sources of energy and certain nutrients as well as temperature changes in natural environments and in the in vitro cultures. Cyanobacteria are known to store carbohydrate as glycogen, phosphate as polyphosphate and nitrogen as multi-L-arginyl-poly (L aspartic acid) or cyanophycin. Little is known about the lipid reserve of cyanobacteria. PHB has been demonstrated in wide range of classical bacteria, but has been reported only for few cyanobacteria [82–84].

Cyanobacteria are also able to accumulate a variety of putative reserve materials. These include cyanophycin, polyphosphate, polysaccharide and PHB. Polysaccharide and PHB are usually considered to

Table 1
PHAs and corresponding R-groups.

R-group	Full name	Short name
CH_3	poly (3-hydroxybutyrate)	PHB
CH_2CH_3	poly (3-hydroxyvalerate)	PHV
$CH_2CH_2CH_3$	poly (3-hydroxyhexanoate)	PHHx

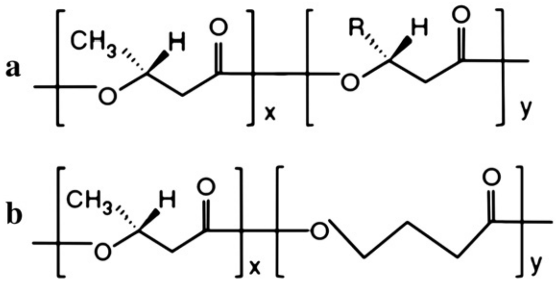

Fig. 2. (a) Poly-β-hydroxybytyrate copolymers. (b) Poly (3-hydroxybutyrate-co-4-hydroxybutyrate) (PHB4B).

Table 2
Physical properties of biopolymers.

Properties	PHB	PHBV	PHB4B	PHBHx	PP
Melting temperature (°C)	177	145	150	127	176
Glass transition temperature (°C)	2	−1	−7	−1	−10
Crystallinity (%)	60	56	45	34	50–70
Tensile strength (MPa)	43	20	26	21	38
Extension to break (%)	5	50	444	400	400

be carbon and energy reserves. In which polyphosphate is a potential energy or phosphate reserve and the polypeptide cyanophycin is, in principle, a nitrogen reserve with a possible additional minor role of supplying limited amounts of carbon and energy [85,86].

4.2. Detection and analysis of poly-β-hydroxybutyrate

While isolating PHB accumulating cyanobacteria from nature, it is necessary to screen rapidly a wide collection of bacteria in a short time. Stains specific to PHB are made use of in the detection of the granules. Viable colony staining technique has been suggested as a method for rapid screening of PHB accumulating bacteria. Sudan black B was first suggested to test cyanobacterial fat stain subsequently. The greater value of this dye was known and the procedure was modified for demonstrating intracellular fatty material in cyanobacteria by preparing microscopic slides and stained with alcoholic Sudan black B solution and counterstained with safranin [87–91]. Poly-β-hydroxybutyrate granules exhibited a strong orange fluorescence when stained with Nile blue. A Heat fixed cells were treated with 1% Nile blue A for 10 min and the excitation was observed at an excitation wavelength of 460 nm. Glycogen and polyphosphate did not stain. Nile blue stain appeared to be a more specific stain for poly-β-hydroxybutyrate than Sudan black B. [92–94]. Screening with Nile red dissolved in acetone distinguished between PHB producers and non-producers. The recommended use of a sensitive viable colony staining method using Nile red for direct screening of cyanobacteria that accumulate PHB has been predominantly used for PHB detection [95–98]. Further the PHB producers exhibited strong fluorescence when observed under UV light.

4.3. Biodegradability and biological considerations of poly-β-hydroxybutyrate

The property that distinguishes PHB from petroleum based plastics is their biodegradability. Biodegradation of PHB under aerobic conditions results in CO_2 and H_2O, where as in anaerobic conditions, the degradation products are CO_2 and CH_4. PHB is compostable over a wide range of temperatures, even at a maximum of around 60 °C with moisture levels at 55%. Studies have shown that 85% of PHA were degraded in seven weeks. PHA has been reported to degrade in aquatic environments (Lake Lugano Switzerland) within 254 days even at temperature not exceeding 60 °C [99–102]. The degradation of PHA by *Ralstonia eutropha* could occur simultaneously with its biosynthesis under nitrogen limitation. This observation is called “cyclic nature of PHA metabolism”. The author reported that the composition of polymer was changed from PHB homopolymer to PHB 49% PHV copolymer when the substrate was changed from butyric acid to pentanoic acid. PHB differed in density from other conventional plastic materials. Because of high density, PHB does not float in an aquatic system. Therefore, once discarded, plastic materials made from PHB will sink and will be degraded in the surface sediment by biogeochemical mechanisms. PHB, P (HB-HV) and other PHA are utilized by microorganisms as an energy source. P (HB-HV) is biodegraded in microbial active environments [103–106]. Microorganisms colonize on the surface of the polymer and secrete enzymes which degrade P (HB-HV) into HB and HV units. These units are then used by the cells as a carbon source for biomass growth. The rate of polymer degradation depends on a variety of factors including surface area, microbial activity of the disposal environment, pH, temperature, moisture and presence of other nutrient materials. P (HB HV) is water insoluble and is not affected by moisture. It does not degrade under normal conditions of storage, and is stable indefinitely in air [107–109].

The end products of PHA degradation in aerobic environments are CO_2 and H_2O, while methane is also produced in anaerobic conditions. The effect of different environments on the degradation rate of PHB and P (HB-HV) has been studied by several workers [110–113]. Degradation occurs most rapidly in anaerobic sewage and slowest in sew water. P (HB-HV) completely degraded after 6, 75and 350 weeks in anaerobic sewage, soil and sea water respectively. PHAs are degraded upon exposure to soil, compost, or marine sediment [114–117]. Biodegradation is dependent on a number of factors such as microbial activity of the environment and the exposed surface area, moisture, temperature and pH. Fig. 3 shows the biodegradability process of poly-β-hydroxybutyrates.

5. Cyanobacterial bio plastics

Cyanobacterial bio plastics manufactured using biopolymers derived from two ways like biopolymers from living organism and polymerizable molecules [118–120]. Biopolymers from living organisms are typically made from cellulose, starch and soy protein. Polymerizable molecules are typically made from lactic acid and triglycerides. Cyanobacterial (blue green algae) based plastics have been a recent trend in the era of bio plastics compared to traditional methods [121–125]. Algae based plastics are in their infancy; once they are into commercialization they are likely to find applications in a wide range of industries. Cyanobacteria serve as an excellent feedstock for plastic production owing to its many advantages such as high yield and the ability to row in a range of environments [126,127]. The use of cyanobacterial plastics opens up the possibility of utilizing carbon, neutralizing greenhouse gas emissions from factories and power plants [128–130]. This provides the double advantages like conservation of fossil resources and reduction in carbon dioxide emissions, which makes an important innovation of sustainable development [131–133].

6. Production and improvement in the commercial PHB producing cyanobacteria

6.1. Genetically engineered cyanobacteria that can accumulate PHB

Genetically engineered cyanobacteria that accumulate PHB were transformed with the genes encoding PHB synthesis (3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase) [134–137]. Metabolic engineering is being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHB synthesis and to produce novel PHB in cyanobacteria. However, this area is totally new as far as cyanobacteria for PHB production and only few reports are published in this direction. Various species of cyanobacteria accumulate considerable amounts of PHB and the non PHB accumulating cyanobacteria have recently been genetically engineered by heterologous transformation with genes involved in the PHB pathway of *R. eutropha*, leading to the accumulation of the polymer. To develop an efficient system for PHB production by cyanobacteria, the genetic characterization of PHB accumulating cyanobacteria was studied in detail [138,139]. The production of pigment free PHB granules by genetically engineered cyanobacteria was isolated from the species *R. eutropha*. Bacteria such as *Escherichia coli* are incapable of synthesizing or degrading PHB [140–142]. However, *E. coli* grows fast, even at higher temperature and is easy to lyse. Faster growth will enable it to accumulate a large amount of polymer. The easy lysis of the cells saves the cost of the purification of PHB granules [143–145]. Hence, *E. coli* has been used to transfer PHB genes. PHB production has been studied mostly in recombinant *E. coli* cells harboring PHB synthesizing genes from *R. eutropha* [128,146–148].

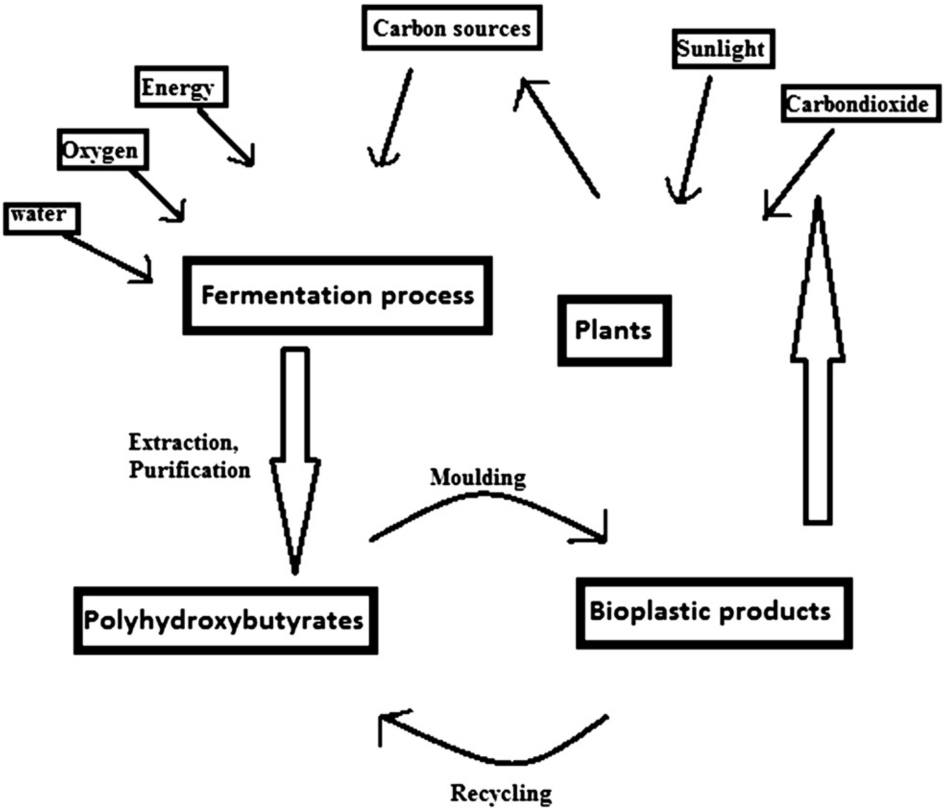

Fig. 3. Biodegradability process of poly-β-hydroxybutyrates.

6.2. Economic status of bio plastics from cyanobacteria

Biotechnological approaches like fermentation and genetic engineering play a key role in conducting the feasibility and sustainability studies in algae bio plastics. The plastics market is worth more than $400 billion and the contribution made by the bio plastics is meager, the key reason for the minor contribution is its high cost. The various plastics that can be made from algae feedstock are hybrid plastics, cellulose based plastics, poly lactic acid and bio polyethylene [149–152]. These plastics from algae are technically feasible since and their economic feasibility is still being working out.

7. Future prospects of cyanobacterial PHB in the industry and needed research

The expectations in this century for polymeric materials demand are, in favor of 2 to 3 fold increase of polymeric material production as a consequence of the increase in their consumption especially in the developing countries. In this approach it is expected that the materials made of biodegradable polymers stay stable during processing, storage and use, but undergo degradation under environmental condition during composting [153–156]. Some of the most recent advances in biopolymer research have focused on the genetic engineering of conventional plants, in order to develop molecular processes by which the plants actually produced usable polymer materials with in the cellular tissues. This fully biodegradable thermoplastic polyester material commonly found in nature, as intracellular deposits in bacteria and in cyanobacteria formed as a carbon and energy storage mechanisms when both are under stress, as explained by Geoffrey Coates [157,158]. There is a conventional method of PHB production, which involves energy intensive and expensive process of sugar fermentation.

However, select researches have effectively found methods of which the genes required for PHB production can be engineered into plants which are already commonly produced by crop producers. Progress in the genetic engineering of plants has seen great success in recent years, but public opinion of this practice is mixed. While complete utilization of plants (for both consumption and industrial application) is seen as an ideal goal by many scientists. This basically concludes in favor of the proposal for developing biodegradable plastics [159–162]. But it would need to be one with high environmental performance, as well as achieving new standards of functionality in material performance.

8. Conclusion

Cyanobacteria do have the potential to produce biopolymers like PHB from CO_2 as the sole carbon source, and the yield of PHB could be increased by various means such as nutrient limiting conditions, stress conditions, different PHB enhancing precursor's in vitro etc. The technology routes for the production of algae based bio plastics that are still under the research phase and are far from commercialization. Algal based bio plastics can play a vital role as an environment friendly and biodegradable alternative compared to conventional plastics. However, it is very clear that our view should be green environment to up hold our future generation to be free from plastic pollution, where the PHB producing cyanobacteria contributions are going to be phenomenal.

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