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Polyhydroxyalkanoates from plants and microorganism

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Abstract

A Non-degradable plastic waste is a growing concern because materials made from synthetic polymers are not biodegradable and properly discarded. Due to slow degradability and increasing water, land pollution issues have led to concern about plastics and this normally derived from petroleum based plastics. Plants and microorganism many species naturally produce macromolecule polyesters and are being considered as a replacement for conventional plastics. Polyhydroxyalkanoates (PHAs) production in plants and microbe can supply a sustainable source of bio-plastics and producing polyhydroxyalkanoates as storage materials. Polyhydroxyalkanoates (PHAs) are a class of polymers with various chain lengths and these qualities of polyesters make them functionally comparable to many of the petroleum based plastics. But petroleum derived plastics take several years to degrade however number of microbe can fully degrade PHAs within a year. Biodegradation of PHAs results in carbon dioxide, water and which return to the environment. In recent years various methods and promising strategies involve genetic engineering of microorganisms and plants to introduce production pathways. Now PHA production commercially from plants appears to be a realistic goal for the future. In this review, we highlight PHA properties and PHAs mass production from plants and microbe.

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Introduction

In the recent years accumulation of plastic wastes has become a major concern, this is due to human population increases day by day this lead to the accumulation of huge amounts of non degradable waste materials and damage environment quality. Plastics are man made long chain polymeric molecules (Scott, 1999). Synthetic plastics are extensively used in packaging of products like food, pharmaceuticals, cosmetics, detergents and chemicals. Plastics are resistant against microbial decomposition, their short time of presence in nature could not design new enzyme structures capable of degrading synthetic polymers (Mueller, 2006). Petroleum ether polyester has not only takes much time to decompose however, produce toxins during the degradation process (Thompson *et al.,* 2004). In 1980s scientists started to look plastics production of materials that can be readily decomposed as well as linked to diminishing petrochemical reserves (Gross and Kalra, 2002). The present industrial world is greatly dependent on fossil fuels as a source of energy; however, fossil fuels are a limited resource (Murray and King, 2012). Globally economy is dependent on oil and recent evidence suggested that discovery of fossil fuel reservoir limited. Approximately 140 million tons plastics consume per annum and processing of plastics use 150 million tons of fossil fuels. Recent challenge is we can substitute the source of these long carbon arrays from a non-sustainable source with a sustainable renewable one. Due to comparable material properties to conventional plastics the biodegradable plastics (polyesters), namely polyhydroxyalkanoates (PHA), polylactides, aliphatic polyesters, polysaccharides and copolymer have been developed successfully over the last few years. Biopolymers obtained from microorganisms and from plants which are genetically-engineered to produce such polymers are likely to replace currently used plastics at least in some of the fields. Biopolymers that are synthesized and catabolized by various organisms and do not cause toxic effects and have certain advantages over petroleum derived plastic (Steinbüchel, 2005; Tokiwa and Calabia, 2004; Reddy *et al.*, 2003; Jendrossek and Handrick, 2002). PHAs biopolymers gather as storage materials in microbial cells under stress conditions (Keshavarz and Ipsita, 2010). PHA production in microbe requires sterile condition in a costly fermentation process. In contrast, plant system PHA production is considerably less expensive as the system only depends on water, soil nutrients, atmospheric CO2 and sunlight (Suriyamongkol *et al.,* 2007).

The mainly produced bio-plastics from microbe and plants are polyhydroxyalkanoates (PHAs) and their derivatives. Composition of PHAs was first described by Lemoigne (Lemoigne, 1927) and poly-3-hydroxybutyrate (PHB) is the simplest form of the polyhydroxyalkanoate (PHA) family of microbial carbon and energy storage materials. This natural thermoplastic polyester and its variants have properties similar to petroleum based plastics yet are biodegradable and number of Scientists reported the production of PHB in Arabidopsis thaliana (Poirier *et al.,* 1992). In 1958 by Macrae and Wilkinson were first reported functions of PHB (Macrae and Wilkinson, 1958). PHB and other forms of PHAs found living organism and the possible use of these biopolymers was started (Keshavarz and Ipsita, 2010). PHAs are an ideal value-added co-product for crops as they possess properties making them suitable replacements for many of the high volume plastics that are currently produced from petroleum resources. By current review we begin a discussion about properties of PHAs and followed by an examination of PHA synthesis in microorganisms and production of PHAs in plants.

Properties of Polyhydroxyalkanoates

PHAs composed of 3-hydroxy fatty acid monomers and which form linear head-to-tail polyester. Normally PHAs accumulation occurs when the cells experience a nutrient imbalance and function as a reservoir of stored carbon that can be degraded when needed by a wide range of microorganisms capable of expressing intracellular or extracellular de-polymerase enzymes (Jendrossek and Handrick, 2002). This feature makes PHAs degradable in all

biologically active environments and PHAs can have different monomer units. Three broad classes of PHAs according to the size of comprising monomers. If PHAs containing up to C5 monomers are classified as short chain length PHAs and when PHAs with C6–C14 and aboveC14 monomers are classified as medium chain length and long chain length, respectively (Suriyamongkol *et al.,* 2007). PHAs polymer final chain length is determined by both the carbon source that is available to the organism and the substrate specificity of the host organisms. PHAs extracted from bacterial cells similar to polypropylene. In plants, exceptional pathways must be engineered to allow conversion of existing plant metabolites to monomers that can be polymerized by a PHA synthase. PHAs can be degraded by many microorganisms at a high rate into carbon dioxide and water. PHAs can be produced from renewable, recyclable resources and large number monomer in PHAs provides a wide variety of polymers with different physical properties.

In recent years PHB has received the most attention as a target molecule for bio-plastic production in plants. PHB is a very hard and brittle material so has some limitations use as a plastic. PHB similar to compostable plastic poly lactic acid (PLA) made through chemical polymerization of lactic acid produced from a fermentation process. PLA material is fairly low cost, and its uses in the plastic market have expanded based on the development of additive materials which improve its properties (Sherman, 2010). If we add other materials and additives in the PHB as a result applications of PHB can also be expanded (Chen and Luo, 2009; Snell and Peoples, 2009). A number of the scientists found that PHB is likely to be the first PHA to be produced directly from crops. PHAs have mainly been used as biodegradable bio-plastics, but unique features of these polymers will allow their use in other applications. Such as heating PHB or biomass containing PHB under the appropriate thermolysis conditions can break the polymer chain releasing the chemical intermediate crotonic acid, which is easily recovered (van-Walsem *et al.,* 2011). Even though a small amount of crotonic acid can be transformed using existing chemistries to a number of drops in commodity chemicals, including propylene by decarboxylation (Peterson and Fischer, 2010) and butanol by hydrogenation. The ability to sequester PHAs in a cell as an inert granular material makes it possible to produce large amounts of a readily convertible polymeric chemical precursor in a biological system where accumulation of the desired chemical itself might be toxic to the host. This broadens the range of renewable chemicals that can be produced from engineered plant feed stocks, and thermolysis provides a simple, scalable, highly efficient recovery option. PHB can also be used as a feed supplement and has been shown to have nutritional value and prebiotic effects (Najdegerami *et al.,* 2012; Boon *et al.,* 2010; De Schryver *et al.,* 2010). In addition to their biodegradability, many PHAs are also biocompatible and PHAs breakdown products are 3-hydroxyacids. Which normally found in animals and can be very useful in many medical applications, such as suture filaments, osteo synthetic materials and a matrix material for slow release drugs and in vitro cell cultures.

PHA synthesis from Microorganisms

Polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are bio-based materials used as substitute of synthetic plastics (Steinbuchel, 2001). Polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received more attention as its bio-degradable properties. Bacteria especially members of the family Halobactericeae of Archaea synthesize PHAs and recently such microorganisms number is increasing which capable to produce PHAs (Berlanga *et al.,* 2006). PHAs synthesis is normally in bacteria under unfavorable conditions and some bacteria reported capable to produce PHA up to 90% (w/w) of dry cells through depletion of essential nutrients such as nitrogen, phosphorus or magnesium (Madison and Huisman, 1999). In bacteria PHA serves as storage compounds but also as a sink for reducing equivalents for some microorganisms. PHA acts as an ideal storage compound because it's insoluble in bacterial

cytoplasm and exerts insignificant increase in osmotic pressure.

PHAs chemical variety is huge and the most widely produced form of PHAs is PHB (Kim and Lenz, 2001). In the synthesis of PHB involved three enzymes such as *phaA* gene encodes β-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. Reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA is catalyzed by the acetoacetyl-CoA reductase. The enzyme is encoded by the *phaB* gene and is NADPH-dependent and polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase, which is encoded by the *phaC* gene. PHA synthase in *Ralstonia eutropha* involves limited array of substrates about C3–C5 chain length (Elsayed *et al.,* 2013; Reddy *et al.,* 2003; Steinbüchel and Schlegel, 1991). So PHAs synthesis through this pathway contains short chain length monomers and three enzymes for PHB synthesis are mainly located in the cytoplasm of the cell. In this process the condensation of propionyl-CoA with acetyl-CoA is mediated by a distinct ketothiolase (Dias *et al.,* 2006; Luengo *et al.,* 2003). Acetoacetyl-CoA reductase and PHA synthase catalyze reduction of 3-ketovaleryl-CoA to (R)-3-hydroxyvaleryl-CoA and consequent polymerization to form HB–HV type PHAs (Poirier, 2002). PHAs synthesis isolated from different bacteria are able to produce PHAs using an extensive range of hydroxyacyl-CoA thio esters as substrates.

PHA synthesis is organized into four classes according to their substrate availability and their subunit compositions. PHAs synthesis encoding genes have been cloned as more than a few natural producers of the biopolymer (Hai *et al.,* 2004). First phaA gene was cloned from *Zoogloea ramigera* using anti-thiolase antibodies (Ueda *et al.*, 1996). In R. eutropha, Acinetobacter spp., *Alcaligenes latusand Pseudomonas acidophila pha* genes form a phaCAB operon even though these three genes are not in the similar sequence in these species. In *Chromatium vinosum*, *Thiocystis violacea*, *Thiocapsa pfennigii* and Synechocysti ssp. PCC 6803 PHA synthase consists of two sub-units such as *PhaC* and *PhaE*. Type III synthase usually catalyzes the synthesis of scl-PHAs, but sometime catalyzes the polymerization of scl and mcl monomers (Steinbüchel and Hein, 2001). Additional genes encode other enzymes that indirectly contribute to PHA synthesis. The PHA synthase gene phaC in *Aeromonas caviaeis* flanked *phaJ*, which encodes enoyl-CoA hydratase. R. eutropha, *Burkholderia caryophylli*, and *Pseudomonas aeruginosa* are able to form mcl-PHAs. The phaC1ZC2D operon in microorganisms have two *phaC* genes, which encodes a PHA depolymerase and the role of PhaD still unclear though it's required for PHA formation (Hang *et al.,* 2002). PHA synthesis in *P. aeruginosa* directly uses intermediates from the fatty acid β-oxidation pathway to form larger molecules of 3-hydroxyacyl-CoA (Luengo *et al.,* 2003). The PHA synthase of *Pseudomonas oleovoranscan* also catalyze the polymerization of a wider range of monomers, which results in higher molecular weight polymers with better elastic properties. Numerous microorganisms also carry an additional cluster (*phaFI*) located downstream from the phaC1ZC2D operon (Nishikawa *et al.,* 2002). *PhaI* participates in the formation and stabilization of the granules, while *PhaF* is involved in the stabilization of the granules and acts as a regulator (Luengo *et al.,* 2003). Over expression of phage genes in the natural PHA producer resulted in little difference in polymer accumulation. A natural producer such as *R. eutropha* is well adapted to PHA accumulation in their cells and store up to 90% of its dry weight. Most natural producers though take a long time so these PHA producers are not suitable for industrial production. Even though *Escherichia coli* do not naturally produce PHA but considered to be suitable host for generating higher yields of the biopolymer as its fast growth and no difficult to lyses (Li *et al.,* 2006). Yet wide attempts at maximizing PHB production but PHB accumulation level were not as high as what could be obtained with the natural producers of the biopolymer. A main impediment in producing PHB in recombinant organisms is associated with the instability of the *pha* genes but other parameters have been studied to enhance PHB

production such as increased carbon supply, changes in fermentation temperature, changes in the number of plasmid copies and choice of bacterial strains. (Nikel *et al.,* 2006). The molecular mass of the PHB produced in E. coli cells depended strongly on culture condition (Kusaka *et al.,* 1997).

PHA production from plants

In bacteria production of PHAs is expensive but PHAs production in plants may be an attractive alternative. Plants are ideal for synthesizing PHAs as they have been shown to be active producers of biomass compared to bacteria. *Ralstonia eutropha* accumulate up to 85 % of dry weight commodities which may PHB and starch respectively. Recent studies demonstrate that genetically engineered plants have ability to produce foreign proteins that are biologically active (Ma *et al.,* 2005). Plants are able to fix carbon dioxide sources through photosynthesis, so plants are efficient to use direct carbon source for PHA production by eliminating the carbon input cost. The cost of PHAs production from plants may comparable to petroleum-based plastics as no cost of feedstock and fermentation settings incase of bacteria (Yunus *et al.,* 2008). Acetyl-CoA is precursor to the PHAs biosynthetic pathway and acetyl-CoA is present in the cytoplasm, plastid, mitochondria and peroxisome of plant cell. The synthesis of PHAs achieved in any of these organelles and high storage space available. PHB production from plants requires genetic engineering of *phbA*, *phbB* and *phbC* genes of *Ralstonia eutropha* and PHBv production needed bktB, phbB, phbC genes of *Ralstonia eutropha* and *ilvA* gene of *Escherichia coli*.

Arabidopsis as a model plant plays a crucial role in the understanding of other plant genes and their biological functions. Arabidopsis was the first plant used as the model for the production of PHAs in plants. Arabidopsis reported to produce PHB in the cytoplasm and PHB has been the target of most of research it requires the coordinated expression of only three genes to convert acetyl-CoA into polymer (Madison and Huisman, 1999). Acetyl-CoA is present at different compartments of plant cells and cellular membranes are impermeable to acetyl-CoA so each organelle has distinct metabolic pathways for Acetyl-CoA synthesis. In photosynthesis and cellular respiration Acetyl-CoA is key metabolite and an intermediate in the biosynthesis of a wide range of secondary metabolites (Fatland *et al.,* 2005). The flux of acetyl-CoA is mainly high in plastids where Acetyl-CoA used in fatty acid synthesis for membrane lipids in leaf chloroplasts and for storage lipids in plastids of seeds. Two molecules of acetyl-CoA that are converted to acetoacetyl-CoA and one NADPH is required for the consequent conversion of acetoacetyl-CoA to monomer R-3-hydroxybutyryl-CoA by a reductase and PHB synthase add the monomer into the growing polymer chain. Different techniques for engineering the PHB genes not only focused on the presence of acetyl-CoA in the targeted compartment, but also on the potential effects of diverting acetyl-CoA and NADPH away from the natural plant metabolism. Scientist succeeded in producing co-polymers of short and medium chain-length PHAs by diverting carbon away from other pathways (Suriyamongkol *et al.,* 2007).

In the Arabidopsis plant PHB production genes are inserted to express a reductase and a PHA synthase from *Cupriavidus necator* from cauliflower mosaic virus (Poirier *et al.,* 1992). C_3 plants shown little success PHB engineered genes in the cytosol and small amounts of polymer produce regularly in the host plant. The highest levels of PHAs produced in the cytosol of Arabidopsis engineered with a mutant, highly active PHA synthase (Hempel *et al.,* 2011). Scientists trying to produce PHB in chloroplasts through Agrobacterium mediated transformation in Arabidopsis. Each transformed construct comprised of an expression cassette for thiolase, reductase synthase from *Alcaligenes eutrophus*. The PHB coding gene was modified at the 5′ end with a DNA fragment encoding the transit peptide from the small subunit of Rubisco from pea to target the encoded enzymes to the plastids. PHB gene expression was found to be higher in older leaves than in younger leaves. (Nawrath *et al.,* 1994). PHB production in plastids used multi-gene constructs containing the

three PHB genes in individual expression cassettes. Plant lines obtained after transformation of the multi-gene constructs yielded plants with a maximum PHB content and number of scientists generated Arabidopsis lines producing up to 4% PHB using a multi-gene vector (Bohmert *et al.,* 2000; Valentin *et al.,* 1999). PHB pathway genes expression from inducible promoters has been used as a plan to reduce the effects of high PHB levels on plants by allowing an initial period of normal plant growth prior to induction of polymer synthesis (Kourtz *et al.,* 2007). Other researcher efforts to produce PHB in plastids have included expression of the transgenes from the plastome of tobacco the plant which is most willingly engineered by plastid transformation. Expression of transgenes from the plastome can give high levels of protein accumulation and which can be controlled to some amount by the choice of 5′ and 3′ un-translated regions flanking trans-genes (Leossl *et al.,* 2005). Recently, researcher work on transgenes encoding the thiolase and synthase from Acinetobacter spp. Reductase from *Bacillus megaterium*, were flanked with UTRs known to give high levels of transgene expression. Scientists observed that plant with up to PHB18.8% DW in leaf samples and 8.8% DW in a whole plant. Peroxisomal b-oxidation pathways are most active and therefore capable of supplying the most substrate for polymer synthesis in young seedlings. Early work to produce PHAs in peroxisomes focused on the production of PHAMCL by expressing a PHA synthase with substrate specificity (Poirier, 2002). Peroxisomal PHB synthesis genes are engineered in Arabidopsishas from R. *eutropha* (Tilbrook *et al.,* 2011).

C_4 plant biomass production is high and makes them model for the production of PHAs mainly nonfood crops because that can grow well on marginal land that is not considered prime food production acreage. C_4 plants photosynthesis rate is mostly high and C_4 plants mainly include grass family such as sugarcane, maize, sorghum, switch grass, energy cane, sweet sorghum and pearl millet. C_4 plants are better than C_3 plants because in C_3 plants, carbon fixation pathway under conditions promoting photorespiration due to the mechanism of CO_2 concentration around Rubisco allowing the enzyme to reach its maximal catalytic activity. Mostly C_4 plant is achieved high CO_2 concentration by the spatial separation (C4cycle) and reduction (Calvin cycle) in two highly specialized leaf cell types such as mesophyll and bundle sheath. In leaves these cells are arranged around the vascular bundles in a sheath-like structure known as Kranz anatomy and are connected to each other through cytoplasmic strands. Capture of CO_2 occurs in C4 plants in the outer ring of mesophyll cells and fixed carbon is transferred in the form of a four-carbon molecule in the bundle sheath cells where CO_2 is released by decarboxylase enzymes. In this way Rubisco oxygenase activity leading to photorespiration is suppressed by high CO_2 concentrations which are estimated to be three to eight times higher than those in C_3 photosynthetic cells (Kanai and Edwards, 1999). As increased photosynthetic rate (up to tenfold higher CO_2 assimilation rates than the most productive C_3 plants), C_4 plants use water and nitrogen more efficiently than C_3 species (Ehleringer and Monson, 1993) but complex compartmentalization of C_4 photosynthesis in bundle sheath and mesophyll cells though creates problems for uniform accumulation of an engineered bio-product such as PHAs. It is also difficult to compare and predict product yields in plants belonging to the different subtypes of C_4 photosynthesis [i.e. NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME) and phosphoenolpyruvate carboxykinase (PCK)] with biochemically distinct steps for the release of CO_2 in the bundle sheath cells. In the engineering of C_4 plants for PHA production has included *Zea mays* L. and Saccharum spp. Which both possess the NADP-ME subtype of C_4 photosynthesis, and *Panicum virgatum* L., an NAD-ME plant. Engineering of PHB production in maize was the first explanation of bioplastic production in a C_4 crop. Genes encoding the PHB enzymes from *R. eutropha* were assembled in multi gene transformation vectors and different viral and plant promoters were used to drive their expression (Mitsky *et al.,* 2003). Increase transgene expression all promoter sequences were

fused to the maize heat shock protein 70 introns.

Higher levels of PHAs polymer were produced in older leaves as compared to younger leaves in Arabidopsis (Nawrath *et al.*, 1994), sugarcane (Purnell *et al.*, 2007), switch grass (Somleva *et al.,* 2008) and tobacco (Bohmert-Tatarev *et al.,* 2011). Poirier and Gruys, (2002) was explained a correlation among higher levels of PHB production and leaf chlorosis and PHB production in chloroplasts. Saccharum spp. was transformed for the production of PHB through inserting multiple single gene vectors to express the genes from R. Eutropha encoding PHB biosynthetic enzymes (Petrasovits *et al.,* 2012). *Zea mays* were used polyubiquitin promoter (ubi1) to drive the transgene expression and encoded enzymes were targeted to the plastids using the RbcS-TP from pea. Single gene vector was introduced at once into sugarcane callus cultures by particle bombardment and 130 transgenic plants screened produced polymer at levels notice able up to 0.01% DW was detected in stems. In Saccharum spp. plastids bundle sheath cells contain PHB granules, but were not visible in mesophyll plastids (Petrasovits *et al.,* 2007). Number of Scientists suggested that the maize ubi1 promoter has previously been depicted to drive the expression of trans-genes in the mesophyll cells of sugarcane and maize. Uneven product distribution might be due to inefficient transport of enzymes to plastids of mesophyll cells with a C_3 dicot chloroplast targeting signal or an inability to efficiently produce PHB in cells involved with the light reactions of photosynthesis (Petrasovits *et al.,* 2007).

Conclusion

Recent researches indicate that production of PHAs as petroleum based plastic alternatives for the future using bacterial and plant systems. Protein engineering of PHA synthases advances made possible to alter the properties so as to produce PHAs with desired monomer composition. Bacterial fermentation depends on external carbon sources such as glucose, but in plants synthesis of PHA is more cost effective technique because plant relies on their own carbon dioxide and light. In plant systems several ingenious approaches have been used to capture intermediates of carbon catabolism and convert them into PHA. Different compartments of the plant cell and different tissues of the plant have been examined for their suitability in producing and storing PHA granules.

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