*I Research Journal of Environment Sciences*_____ ISSN 2319–1414
Vol. **1(2),** 46-52, September **(2012)** *I. Res. J. Environment Sci.*

Review Paper

PHA - Production Application and its Bioremediation in Environment

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Available online at: www.isca.in
Received 14th July 2012, revised 18th July 2012, accepted 24th July 2012

Abstract

The research article covers the occurrence of and biosynthesis of PHA's. Various PHA production methods are discussed, PHA production by recombinant bacteria, transgenic plants and the application viz. industrial application, agriculture application have been highlighted. Biodegradation of PHA compounds in the environment has also been listed. The paper will be beneficial to readers to understand PHA production, application and their degradation in the environment.

Key words: PHA, Recombinant bacteria, transgenic plants, bioremediation

Introduction

Global dependence on petroleum-derived plastics has increased considerably over the years. However, the rapid depletion of crude oil and the mounting apprehension about the environmental effects of synthetically produced materials has prompted much interest in biologically derived polymers, particularly of the biodegradable class. Modern biotechnology has made it possible to manipulate these biopolymers to suit human needs. Among the various biodegradable polymers, a class that is drawing considerable attention is the polyhydroxyalkanoates. Polyhydroxyalkanoates (PHAs) are a family of linear polyesters of 3, 4, 5 & 6-hydroxyacids, synthesized by a wide variety of bacteria through the fermentation of sugars, lipids, alkanes, alkenes andalkanoic acids. They are found as discrete cytoplasmic inclusions in bacterial cells. Once extracted from the cells, PHAs exhibit thermoplastic and elastomeric properties. PHAs are recyclable, are natural materials and can be easily degraded to carbon dioxide and water. Hence they are excellent replacements for petroleum-derived plastics in terms of process ability, physical characteristics and biodegradability. In addition, these polymers are biocompatible and hence have several medical applications. All of the monomeric units of PHAs are enantiomerically pure and in the R-configuration. R-hydroxyalkanoic acids produced by the hydrolysis of PHAs can also be widely used as chiral starting materials in fine chemical, pharmaceutical and medical industries.[1, 4,15,16,17]

Occurrence and Biosynthesis of Phas: A wide variety of bacteria both Gram negative and Gram positive such as *Pseudomonas, Bacillus, Ralstonia, Aeromonas, Rhodobacter* and certain Archaea, especially members of the *Halobactericeae*, like *Haloferaxsulfurifontis*, synthesise-polyhydroxyalkanoates. They function as energy storage compounds and are present in the cells as insoluble granules in the cytoplasm. Marine prokaryotes, both bacteria and archae, also produce PHAs that have tremendous commercial potential. PHAs accumulate up to 80% dry cell weight in these organisms when present in a 'high-nutrient econiche'. This displays the widespread occurrence of PHA-producing microbes in the environment. Polyhydroxy-alkanoates (PHAs) are divided into two groups based on the number of constituent carbon atoms in their monomer units – short-chain length (SCL) PHAs and medium-chain-length (MCL) PHAs. The former consists of monomers with 3–5 carbon atoms and the latter contains monomers with 6–14 carbon atoms. Recently, there have been reports of several bacteria that are able to synthesise PHAs containing both SCL- and MCL-monomer units.[1,8,14] PHASCL are stiff and brittle with a high degree of crystallinity whereas PHAMCL are flexible, have low crystallinity, tensile strength and melting point.

The supply of the substrate monomer and the polymerization of these monomers are the two main steps involved in the biosynthesis of PHAs. The PHA synthesized by a microbe is dependent on the carbon source used. Carbon sources have been classified as 'related' sources that give rise to monomers that are structurally identical to that particular carbon source and 'unrelated' sources that generate monomers that are completely different from the given carbon source. The cause for this difference can be elucidated from the metabolic pathways operating in the microorganism. There are three well-known PHA biosynthetic pathways (Fig.1).Pathway I used by *Cupriavidusnecator* (previously known as *Wauterisiaeutro-phaor Ralstoniaeutrophus*) is the best known among the PHA biosynthetic pathways. In this pathway, 3HB monomers are generated by the condensation of two acetyl-CoA molecules, from the tricarboxylic acid (TCA) cycle to form acetoacetyl-CoA by the enzyme *β*-ketothiolase. Then acetoacetyl-CoA reductase acts on acetoacetyl-CoA to form 3-hydroxybutyryl-CoA. Finally, the PHA synthase enzyme catalyses the polymerization via esterification of 3-hydroxybutyryl-CoA

into poly(3-hydroxybutyrate) (P(3HB)). Pathways involved in fatty acid metabolism generate different hydroxyalkanoate monomers utilized in PHA biosynthesis. The fatty acid *β*-oxidation pathway (Pathway II) generates substrates that can be polymerized by the PHA synthases of *Pseudomonads* belonging to the ribosomal RNA homology group I such as *Pseudomonas aeruginosa*. These microbes can synthesize PHAMCL from various alkanes, alkenes and alkanoates.

The monomer composition is related to the carbon source used. In *Aeromonascaviae*, the *β*-oxidation intermediate, trans- 2-enoyl-CoA is converted to (R)-hydroxyacyl-CoA by a (R)-specific enoyl-CoA hydratase T suge also found the presence of similar enoyl-CoA hydratases in *Pseudomonas aeruginosa*.Huijbert*set al*. showed that PHA synthases (such as that of *Pseudomonas putida*) that catalyse PHA synthesis from fatty acids are also responsible for PHA synthesis from glucose. The intermediates for this channel of synthesis were obtained from the fatty acid *de novo* biosynthetic pathway (Pathway III). Pathway III is of significant interest because it helps generate monomers for PHA synthesis from structurally unrelated and simple, inexpensive carbon sources such as glucose, sucrose and fructose. The(R)-3-hydroxyacyl intermediates from the fatty acid biosynthetic pathway are converted from their acyl carrier protein (ACP) form to the CoA form by the enzyme acyl-ACP-CoA transacylase (encoded by phaG).This enzyme is the key link between fatty acid synthesis and PHA biosynthesis. [47, 1, 13, 16]

PHA production by recombinant bacteria: Natural PHA-producing bacteria have a long generation time and relatively low optimal growth temperature. These are often hard to lyse and contain pathways for PHA degradation. Bacteria such as *E.coli* are incapable of synthesizing or degrading PHA; however *E. coli* grows fast, even at high temperature and is easy to lyse. Fast growth will enable it to accumulate a large amount of polymer. The easy lysis of the cells saves the cost of the purification of the PHA granules Metabolic engineering is being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E. coli* strains harboring the *Alcaligeneseutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high PHA productivity. Since *E. coli* can utilize various carbon sources, including glucose, sucrose, lactose and xylose, a further cost reduction in PHA is possible by using cheap substrates such as molasses, whey and hemicellulose hydrolysate. This strategy can be extended to virtually any bacterium if it possesses metabolic advantages over those currently in use. Heterologous expression of PHA biosynthetic genes of *A. eutrophus* in *Pseudomonas oleovorans* (which synthesizes only medium chain length PHA), has allowed the production of a blend of P(3HB) and msc- PHA The production of various PHA using natural isolates and recombinant bacteria with different substrates is presented in Two approaches can be taken in the development of bacterial strains that produce PHA from inexpensive carbon substrates. First, the substrate utilization genes can be introduced into the PHA producers. Second, PHA biosynthetic genes can be introduced into the non-PHA producers, which can utilize cheap substrates. At present, the second approach seems to be more promising.[47, 1, 17]

PHA production in transgenic plants: Currently, PHAs for commercial applications are being produced by microbial fermentations. However, continuing efforts are being made to devise cost-effective means such as transgenic plants, to produce PHAs. Transgenic plants have always been utilized for producing genetically modified (GM) crops for food purposes and have always attracted suspicion and ethical debates. Producing PHAs in plants, on the other hand, will help connect the low cost/high-volume sustainable production capacity of crops with the vast number of developing polymer industries and should not promote much controversy. This will enable PHAs to possess both superior cost and performance factors. Many research groups have reported their attempts to produce PHAs in plant systems. The only raw materials required will be carbon dioxide for carbon and sunlight as the energy source. The overall costs would make PHA production economical. Transgenic plants containing the PHA synthase genes have been created. The transgenic plants were stunted but accumulated about 15% dcw (dry cell weight) of P(3HB) in the leaf expression systems. Co-polymers of 3HB and 3HV were produced in *Arabidopsis* and *Brassica rapa*. A molecular mass of was attained which is excellent for commercial applications. Nawrath and coworkers attempted the production of PHAs in plastids whereas Poirier's group targeted PHA production in seeds. *Arabidopsis* plants accumulated P (3HB) up to 14% of the leaf dry weight. Transgenic PHA-producing plants have also been produced using tobacco, cotton, and flax systems. However, plant systems have other disadvantages. It is difficult to produce copolymers in plant systems since the production is under the control of endogenous metabolic precursors in the plant. Similarly, the recovery of the polymer from plant tissues is a tricky and expensive affair. Monsanto previously produced poly (3HB) and poly (3HB-co- HV) in *Arabidopsis* and *Brassica rapa*and these polymers resembled the bacterial polymers. Researchers in Japan have also recently devised a transgenic *Arabidopsis thaliana*, harbouring an engineered PHA synthase gene from *Pseudomonas* sp. 3; *fabH*gene (codes for 3-ketoacyl-ACP synthase III) from *E. coli* and a *phaAB*gene (codes for a ketothiolase and acetoacetyl-CoA reductase) from *Cupriavi-dusnecator* and the enzymes were targeted to the plastids. A polymer consisting of 3-hydroxybutyrate unit and a small portion of 3-HA units (C5–C14) was produced. The *fabH*gene aided a two-fold increase in the average PHA content but the maximum PHA amount remained the same and any further increase led to stunted growth. However, the tissue-specific PHA production countered that. The same

group also carried out seed-specific PHA production in rice and tuber-specific production in potato. Purnell*et al*. are actively investigating the accumulation of PHA in sugarcane. PHA biosynthetic genes from *Cupriavidusnecator*were expressed in the plastids. Although the maximum polymer accumulated in leaves was only 0.26%, the characteristic stunted growth associated with transgenic plants producing PHAs was not observed. These set of experiments have definitely enhanced hope in the direction of cheaper PHA production. Metabolix in alliance with British Petroleum has now successfully transferred the PHA metabolic pathway into switchgrass (*Panicumvirgatum*). Switchgrass grows quickly, converting solar energy into chemical energy. Switchgrass also absorbs carbon dioxide from the atmosphere as it grows, thus reducing the buildup of this gas in the atmosphere. They have developed a detailed biorefinery cost and engineering analysis using switchgrass, which is being used to promote large-scale PHA manufacturing. Hence, altered plant phenotypes, low productivity and transgenic stability are problems that have to be resolved before transgenic plants become the chosen mode of PHA production.[18, 47, 1]

Applications: In recent years, the number of research publications dealing with biosynthesis, fermentation, and characterization of the PHA family of biopolymers has increased. What started as an academic interest is now swiftly moving into the commercial field. In 1990, about 25% of the plastics market (about 7.5 million tonnes) was consumed by the packaging industry. In 1993, it was predicted that the demand for biodegradable materials would increase within 3 years and a major portion of that demand would be from the packaging industry. The plastics market in the USA is considerable with production amounting to about 170 million tons per year and expanding at around 4–5% per annum. Initially, PHAs were used tomake everyday articles such as shampoo bottles and packaging materials. The first consumer product made out of PHA was launched in April 1990 by Wella AG. They tested their Sanara range of biodegradable shampoos in bottles made of BIOPOL™ (ICI, UK). Over the last decade, applications have increased both in variety and specialization.[1, 47, 13] A detailed discussion applications follows.

Industrial applications: The use of PHAs in industry has been impressive. PHA latex can be used to cover paper or cardboard to make water-resistant surfaces as opposed to the combination of cardboard with aluminum that is currently used and is non-biodegradable. This also works out be cost-effective since a very small amount of PHAs is required for this purpose. PHAs can be used to make foils, films and diaphragms. Biomer, a German company owns the technology to produce P (3HB) from *Alcaligeneslatus*on a large scale. The cells are grown in a mineral medium using sucrose as a carbon source. The strain used can accumulate up to 90% P (3HB) in dry cell weight. The polymer thus produced is used to make articles such as combs, pens and bullets. The polymer pellets are sold commercially for use in classical transformation processes. The melted polymers have low viscosity, permitting the injection moulding of objects with thin walls. The end product is very hard and can be used at temperatures from 30°C to 120°C. This product degrades within two months in the environment. Metabolix, a US-based company, now markets among others, Metabolix PHA which is a blend of P (3HB) and poly (3-hydroxyoctanoate). This is an elastomer that has been approved by the FDA for production of food additives. Metabolix has created a recombinant *Escherichia coli* K12 strain for this purpose. These cells can accumulate up to 90% of PHA in dry cell weight in 24 h. In 2001, Metabolix was awarded a Department of Energy (DOE) grant of $2 million to develop PHA production directly in switchgrass. Switchgrass, a perennial plant that usually thrives on marginal land, is a leading candidate for biobased production in North America because it can be grown on land of marginal use for other crops. Industrial production of P (3HB-3HHx) by *Aeromonashydrophila*has been accomplished by Tsinghua University (China) in collaboration with Guangdong Jiangmen Center for Biotech Development (China), KAIST (Korea) and Procter & Gamble (USA). The polymer produced is used to make flushable, nonwovens, binders, flexible packaging, thermoformed articles, synthetic paper and medical devices. It is also possible to use PHAs to make the following articles due to their piezoelectric nature: pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, lighters, gas lighters; acoustics: microphone, ultrasonic detectors, sound pressure measuring instruments; oscillators: headphones, loudspeakers, for ultrasonic therapy and atomization of liquids. The gas barrier property of P (3HB-3HV) is useful for applications in food packaging and for making plastic beverage bottles. The same property can be exploited to make coated paper and films which can be used for coated paper milk cartons. P(3HB) or its copolymers can be used to make the non-woven cover stock and the plastic film moisture barriers in nappies and sanitary towels along with some speciality paramedical film applications in hospitals. Polystyrene waste amounts to about 14 million tonnes per year in the USA alone. A research group at the University College Dublin devised a new method to convert polystyrene waste into biodegradable PHAs. First, polystyrene is converted to styrene oil by pyrolysis, in a fluidized bed reactor. The styrene oil is composed of 8% (w/w) styrene and low levels of other aromatic compounds. When provided as the sole carbon source, *Pseudomonas putida*CA-3 converted the styrene oil to medium chain length PHA. Some researchers have warned that since pyrolysis is an 'energy-demanding' process and can generate hazardous wastes, the whole process is not without its demerits. On the other hand, this process could be the optimum way to use up waste polystyrene cost-effectively and efficiently.[47, 1]

Agricultural applications: PHAs have been used as mulch films for agricultural purposes4. In recent years, Procter &

Gamble have produced Nodax™, which can be used to manufacture biodegradable agricultural film. Nodax™is a copolymer containing mainly 3(HB) and smallquantities of MCL monomers. Nodax™ can degradeanaerobically and hence can be used as a coating forurea fertilizers to be used in rice fields or for herbicidesand insecticides. One of the specialized applications of P (3HB-3HV) in agriculture is in the controlled release of insecticides. Insecticides could be integrated into P (3HB-3HV) pellets and sown along with the farmer's crops. The insecticide would be released at a rate related tothe level of pest activity since the bacteria breaking down the polymer would be affected by the same environmental conditions as that of the soil pests. Another use of PHAs in agriculture is in bacterial inoculants used to enhance nitrogen fixation in plants. The bacterial culture used in inoculants preparations for agricultural purposes need to withstand stressful environments. The bacterial cells have to be stored for long periods, and endure desiccation and hot conditions. Inoculants have to possess the ability to sustain high survival rates within the carrier. Therefore research in this area has focused on the addition of elements like nutrients or other synthetic products that can increase the quality of carriers leading to prolonged survival. From studies carried out on *Azospirillumbrasilense*inoculants, it was observed that while carriers may vary, the plant growth promotion outcome was more constant with *A. brasilense*inoculants containing high amounts of intracellular PHA. This was confirmed by field experiments in Mexico with maize and wheat. Better consistency was achieved in increasing crop yield by using peat inoculants prepared with PHA-rich *Azospirillum*cells. Hence, intracellular PHA is of paramount significance for improving the shelf life, efficiency and reliability of commercial inoculants.[47, 1]

Biodegradation: Perhaps one of the greatest advantages that PHAs possess over other biodegradable polymers is their ability to degrade under both aerobic and anaerobic conditions.[23] they can also be degraded by thermal means or by enzymatic hydrolysis. In a biological system, PHAs can be degraded using microbial depolymerases as well as by nonenzymatic and enzymatic hydrolysis in animal tissues. The biodegradability of a polymer is governed primarily by its physical and chemical properties. It has been found that low molecular weight PHAs are more susceptible to biodegradation. The melting temperature is another important factor to be considered when studying biodegradation.[23] as the melting point increases, the biodegradability decreases. With increasing melting temperature, the enzymatic degradability decreases. Tokiwa and Suzuki found that lipases cannot hydrolyse the optically active P(3HB). This could be due to the high melting temperature of the latter (178 ^{o}C). Mochizuki and Hirami explained that biodegradation of solid polymers is influenced by chemical structure (especially functional groups and hydrophilicity–hydrophobicity balance) and highlyordered structures (mainly crystallinity, orientation and morphological properties). Tokiwa and coworkers reaffirmed that crystallinity plays a very important role in biodegradability. They also identified that highly ordered structures, i.e. highly crystalline materials have lower biodegradability. In addition, the microbial population in a given environment and the temperature also contribute to biodegradability in the environment.[47, 1, 12, 23]

Biodegradation in the environment: P (3HB) has been taken as a prototype in the biodegradation studies of PHAs. Micro-organisms from the families *Pseudonocardiaceae, Micromonosporaceae, Thermomonosporaceae, Streptosporangiaceae*and *Streptomycetaceae*predominantly degrade P (3HB) in the environment. In addition, most PHA-producing bacteria are able to degrade the polymer intracellularly. During intracellular degradation, the PHA depolymerase in the cell breaks down P (3HB) to give 3-hydroxybutyric acid. A dehydrogenase acts on the latter and oxidises it to acetylacetate and a β-ketothiolase acts on acetylacetate to break it down to acetyl-CoA. The β-ketothiolase enzyme plays an important role in both the biosynthetic and the biodegradation pathways. Under aerobic conditions, the acetyl-CoA enters the citric acid cycle and is oxidized to CO_2. Very little is known about the intracellular depolymerases since they are always found to be intimately connected to the P (3HB) granules and the overall process is very complex. Extracellular depolymerases degrade polyhydroxyalkanoates present in the environment. The bacteria, algae and fungi present in the environment attack the polymers on the surface. These microbes secrete extracellular enzymes that solubilize the polymer and these soluble products are then absorbed through their cell walls and utilized. The PHA depolymerase enzymes act on the polymer mainly by hydrophobic interactions. Degradation by these depolymerases initially produces oligomers. Some microbes produce an additional dimer hydrolase, which further breaks down the oligomers into the corresponding monomer. These extracellular depolymerases are quite well understood. The rate of biodegradation of PHAs depend on environmental conditions like temperature, moisture, pH, nutrient supply and those related to the PHA materials themselves, such as, monomer composition, crystallinity, additives and surface area.[1,12,47,23]

Table: 1
Production of PHA by various bacteria [47, 1, 13, 16,19,20,24]

Microorganisms	Carbon source	PHA	PHA content(%w/v)
Alcaligenseutrophus	Gluconate	PHB	4
	Propionate	PHB	26-36
	octanoate	PHB	38-45
Bacillus meganterium	Glucose	PHB	20
Klebsiellaaerogenes	Molasses	PHB	65
Methylobacteriumrhodesianum (MB1267)	Fructose/methanol	PHB	30
M.extorques(ATCC55366)	Methanol	P(3HV)	0.002
Psudomonasaeruginosa	Euphorbia	PHA	20-30
P.denitrificanus	Methanol	P(3HV)	0.02
P.oleovorans	Gluconate	PHB	55
	Octanoate	PHB	50-68
	Palm Kernel oil	PHA	37
	Lauric acid	PHA	25
P.putida	Mystric acid	PHA	28
	Oleic acid11-Phenoxyun-decanoic cid	PHA	19
P.Putida BM O1		5POHV	15-35

Table: 3
PHA Production in recombinant bacteria

Strain	Culture	PHA biosynthesis gene sourse	PHA type
E coli XLI- Blue	Fed- batch	*Alcaligenslatus*	P(3HB)
E coli HMS174	Fed-batch	*Ralstoniaeutropia*	P(3HB)
E coli GCSC4401	Cell cycle Fed-batch	*Alcaligenslatus*	P(3HB)
E coli XLI- Blue	Fed-batch	*Alcaligenslatus*	P(3HB-co3HV
E coli RS3097	Fed-batch	*Psudomonasaeuroginosa*	PHA_{MCL}

Table: 4
Summary of PHA Production in transgenic plant[18,47]

Plant species	Sub cellular PHA compartment	Tissue	PHA produced
Arabidopsis thaliana	Plastid	Shoot	P(3HB)
	Plastid	Shoot	P(3HB-co-3HV)
	Peroxisome	Whole plant	PHA_{MCL}
Alfalfa	Plastid	Shoot	P(3HB)
Corn	Plastid	Shoot	P3HB)
Cotton	Cytoplasm	Fiber	P(3HB)
	Plastid	Fiber	P(3HB)
Maize	Peroxisome	Cell suspension	P(3HB)
Potato	Plastid	Shoot	P(3HB)
Rapeseed	Cytoplasm	Shoot	P(3HB)
Tobacco	Cytoplasm	Shoot	P(3HB)

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