ISSN 2229-5518

Biosynthesis of PHA and it's copolymers – a review

Parul Jindal, D.P.Tiwari

ABSTRACT-

To solve the current problem of pollution caused by the continuous use of conventional plastic recent technologies are directed towards the development of production of PHA because of its resemblance with synthetic plastic. Several strains of rhizobium species are isolated from leguminous plants accumulate PHA as cellular and biogreen material. Biogreen material includes polylactic acid (PLA), naturally occuring zein & poly-3-hydroxy butyrate but major interest is towards the energy storage material. Various species of Rhizobium synthesize up to 67% PHA of their cell biomass having varying amount of PHB & PHV. *Bacillus megaterium* synthesize 72.9% PHA as its cell biomass when activated sludge used as carbon source.

Index terms- Biogreen material, Poly-3-hydroxyalkanoates, Rhizobium Sps, Polyhydroxybutyrate, Copolymer, Solvent extraction, [1]Biodegradability

INTRODUCTION: There is a considerable interest in the development and production of biodegradable polymers that exert negligible side effect on the environment. Biodegradable polymers are the class of polymers produced by living organisms such as polylactic acid (PLA), naturally occuring zein & poly-3-hydroxy butyrate replacing the need for polystyrene or polythene based plastic. PHA is known to be accumulated as intracellular inclusions in some bacteria. The material properties exhibited by PHAs ranging from stiff, brittle to rubber like makes it a close substitute for the synthetic plastic. Thus biodegradable polymers or bioplastic are important and interesting areas that are being looked out as alternatives for synthetic plastic. These are a new generation of materials able to significantly reduce the environmental impact in terms of energy consumption and green house effect.

PHA (POLYHYDROXYALKANOATES): PHAs have attracted attention in recent years because PHA polymers are thermoplastic, can be processed on conventional processing equipment, and are, depending on their composition, ductile and more or less elastic. They differ in their properties according to their chemical composition (homo-or copolyester, contained hydroxy fatty acids). They are UV stable, in contrast to other biopolymers such as polylactic acid; its melting point is 175C, and shows a low permeation of water. It is upto 70% crystalline. Its processability, impact strength and flexibility can be increase with a higher percentage of valerate in the material.

PHAs are the microbial polyesters of HAs. PHAs are synthesized by a number of bacteria as cellular and energy storage materials. These are accumulated in the cytoplasm of cells as granules under conditions of nutrient imbalance. Accumulation usually occurs when carbon is in excess and if at least one other nutrient which is essential for growth is depleted.

PHA being thermoplastic polyesters has the potential to replace petrochemical plastics in a majority of applications. The extensive range of physical properties and broadened

- [1] *Author name is currently pursuing PhD from DCRUST,Murthal, Sonipat131001, Haryana, India. Phone no. 9896532383E.mail id- parul_jindal2008@yahoo.com*
- *Guide name is D.P.Tiwari , Dean of chemical engineering department in DCRUST,Murthal sonipat haryana, India .*

performance obtained by compounding and blending is exploited in such applications. Various applications of PHAs have been envisaged which includes molded containers, back sheet of hygiene articles such as diapers, coating agents, packaging materials etc. It is exploited in bulk applications such as coatings, low strength packing, medium strength structural materials, medical temporary implants (such as scaffolding for the regeneration of arteries and nerve axons), and water based latex paints. It is brittle and hence cannot be used in most of the applications. This is overcome by the synthesis of copolymers of PHB with other PHAs. The copolymers may have better thermo mechanical properties than do PHB homopolymers. A wide variety of copolymers of PHA have been isolated from bacteria by varying the combination of carbon substrate for growth.

Various species of bacteria accumulate up to 60% PHA of cell biomass and the copolymers are synthesized in the presence of propionate or valerate along with different carbon sources. The microorganisms accumulated PHA are easily identified by staining with sudan black and nile blue. However reports regarding the development of mutant strains of bacteria for enhanced production of PHA are scanty, although recombinant strains have been constructed for this purpose.

Discovery and historical development of PHA

Poly (3-hydroxybutyrate) [P (3HB)] is the most common PHA and was first described by Lemoigne, a French scientist in year 1925. [1] After that, various bacterial strains among archaebacteria ,[1] gram positive [2,3] and gram negative bacteria [4] and photosynthetic bacteria [5-8] including cyanobacteria [9,10] have been identified to synthesize P(3HB) both aerobically and anaerobically. P (3HB) as a bacterial storage polymer having function similar to starch and glycogen was accepted by the year 1973. [11] Macrae and Wilkinson noticed that *Bacillus megaterium* accumulate P (3HB) homopolymer when the ratio of glucose to nitrogen in the culture medium was high [12] and the subsequent intracellular degradation of P (3HB) occurred in the absence of carbon and energy sources. [13] The opinion that 3HB is the only monomer unit of this polymer has been changed after the discovery of other monomer units as bacterial storage material. [14, 15]

In 1974, Wallen and Rohwedder discovered other monomer units beside 3HB monomer from activated sewage sludge.[16] Among the polymers extracted from the sludge, 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp) monomers existed as the major and minor constituents; respectively.In 2006 Yuan et al reported that accumulation of short chain PHA by the use of waste activated sludge alkaline fermentation liquid as the carbon source. In the year of 1983, 3HHp was reported in *B. megaterium.* [2] In the same year, De Smet and coworkers identified a new monomer, 3-hydroxyoctanoate (3HO) with trace amount of 3HHx from *Pseudomonas oleovorans* when fed with n-octane. [17] From this investigation it was concluded that the production of various PHA monomers was dependent on the substrate fed.

To date, about 150 different monomer constituents of PHA have been found. [18, 14] Witholt and Kessler have compiled the large variety of PHA monomers with straight, branched, saturated, unsaturated and also aromatic structures. [19]

PHA can be classified according to the monomer size. There are two major groups of PHA;

1.Short-chain-length (SCL) PHA with five or less carbon atoms in a monomer, and

2. Medium-chain-length (MCL) PHA with six to fourteen carbon atoms in a monomer.

Cupriavidus necator (formerly known as *Alcaligenes eutrophus* or *Wautersia eutropha*) is a well studied bacterium capable of producing SCL-PHA and it has been identified to produce PHA polymers consisting of 3HB, 3HV and 4HB monomers. [1,20,21] *P. oleovorans* and *Pseudomonas putida* are known to synthesize MCL-PHA consisting of 3HO and 3-hydroxydecanoate (3HD) monomers as major components. PHA has yet to be produced in large scale because the yield is relatively low compared to SCL-PHA. [22] Now special interest is in the polyhydroxyalkanoates with functional groups in side chain that allow further chemical modification, e.g., halogen, carboxyl, epoxy, phenoxy, cyanophenoxy, nitrophenoxy, thiophenoxy, and methylester groups. The length of the side chain and its functional group influence the properties of PHA, e.g., melting point, glass transition temperature, and crytallinity.In 2006 Keen I et al introduced amine functionality on a poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by ammonia plasma treatment and ethylenediamine aminolysis. Carboxylic moiety is introduced in side olefins chains with the use of osmium tetraoxide and oxone (Stigers DJ, Tew GN, 2003).

ISSN 2229-5518

GENERAL STRUCTURE OF PHA

[-O-CH(R)-(CH2)n-CO-]100-30000

n=1 R = hydrogen poly-3-hydroxypropionate
methyl poly-3hydroxybutyrate
ethyl poly-3-hydroxyvalerate
propyl poly-3hydroxyhexanoate
pentyl poly-3-hydroxyoctanoate
nonyl poly-3-hydroxydodecanoate
n=2 R = hydrogen poly-4-hydroxybutyrate
n=3 R = hydrogen poly-5-hydroxyvalerate

BIOSYNTHESIS OF PHA

BACTERIAL STRAINS: Various recombinant strains of bacteria generally used for the production of PHA presented in the table 1.

Table 1. Bacteria used for production of PHA from plant oils and wastes

Strains	**PHA type**	**Substrates**	**PHA content (Wt %)**	**Ref.**
Alcaligenes latus DSM 1124	P(3HB)	Soya waste, malt waste	33, 71	23
Bacillus megaterium	P(3HB)	Beet molasses, date syrup	~50	24
Burkholderia sp. USM (JCM 15050)	P(3HB)	Palm oil derivatives, fatty acids, glycerol	22-70	25
Comamonas testosteroni	MCL-PHA	Castor oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, sesame oil	79-88	26
Cupriavidus necator	P(3HB)	Bagasse hydrolysates	54	27
Cupriavidus necator H16	P(3HB-*co*-3HV)	Crude palm kernel oil, olive oil, sunflower oil, palm kernel oil, cooking oil, palm oil, crude palm oil, coconut oil + sodium propionate	65-90	28
Cupriavidus necator DSM 545	P(3HB)	Waste glycerol	50	29

Recombinant Cupriavidus necator	P(3HB-*co*-3HHx)	Palm kernel oil, palm oil, cru palm oil, palm acid oil
Recombinant Escherichia coli	P(3HB-*co*-3HHx-*co*-3HO	Soybean oil
Pseudomonas aeruginosa IFO3924	mcl PHA	Palm oil
Pseudomonas aeruginosa NCIB 40045	mcl PHA	Waste frying oil
Pseudomonas guezennei biovar. tikehau	mcl PHA	Coprah oil
Thermus thermophilus HB8	P(3HV-*co*-3HHp-*co*-3HN*co*-3HU	Whey
Rhizobium leguminosarum	P(3HB)	Root nodules of leguminous p
R. japonicum NCIM 2002	PHB + PHV	Root nodules of leguminous p
R.sps. NCIM 2226	PHB	Root nodules of leguminous p
R.meliloti MTCC 100	99.6 P(3HB) + 0.4PHV	Root nodules of leguminous p

Among the more than 250 different natural PHA-producers, only a few bacteria have been employed for the biosynthesis of PHA .The polymer production condition differs from bacteria to bacteria.

These microorganisms are capable to synthesis PHAs by using various carbon sources in limiting amount of other nutrients like nitrogen, sulphur and oxygen etc. But the limitation of this method is its high cost and production in limited amount. That's why it is desirable to develop strains that can produce high PHA content in short period of time from simple & inexpensive substrate.

Fermentation Process: Bacteria which are used for the production of PHA can be categorized into two groups depending upon the culture conditions.First category include those bacteria which require excess of carbon and limiting amount of other nutrients like N,P, S & O for producing PHAs. Second category include those bacteria which accumulate PHA during its exponential growth phase.The culture condition require for large scale production of PHA should be taken into consideration. Batch and fed-batch cultivation are widely used for industrial production of PHA. Fed batch cultivation is more efficient than batch because the medium composition can be controlled by substrate inhibition.

EXTRACTION AND PURIFICATION OF THE POLYMERS

The last stage of PHA production involves separating the polymer from the cells. For this solvent of aqueous extraction can be used. In aqueous process, the cell walls are broken and the polymer is then extracted and purified. This process is less expensive but this process reduces polymer molecular weight. For example solvent extraction can produce copolymer weight of 1 million, whereas typical molecular weights of aqueously extracted copolymer are in the range 600,000.

In solvent extraction process, the solvent employed includes chloroform, methylene chloride, propylene carbonate and dichloroethane. Large amount of solvent is required due to high viscosity of PHA; hence this method is economically less profitable.

Sodium hypochlorite is used for the aqueous process. The use of sodium hypochlorite significantly increased PHA degradation, polymer purity greater than 95% is achieved.

An aqueous enzymatic digestion method is also available to solubilise non-PHA cellular materials.[38]

THE POLYMER BIODEGRADABILIY

PHAs are used by microorganisms as an energy source. PHAs biodegrades in microbial active environments. Microorganisms colonize on the surface of polymer and secrete enzymes, which degrades PHA into HA. These units are then used up by the cells as C-source for biomass growth. The rate of polymer biodegradation depends on a variety of factors including surface area, microbial activity of the disposal environment, pH, temperature, moisture and the pressure of other nutrient materials. P (HB-HV) is water insoluble and is not affected by moisture, does not degrade under normal conditions of storage and is stable indefinitely in air. The end products of PHA degradation in aerobic environments are CO2 &H2O while CH4 is also produced in anaerobic conditions. Degradation occurs most rapidly in anaerobic sewage and slowest in seawater. It has been observed that P (HB-HV) completely degraded after 6, 75& 350 weeks in anaerobic sewage, soil& seawater respectively. [36,38]

APPLICATIONS OF PHA

PHA has a wide range of applications because of its biocompatibility, biodegradability
and negligible cytotoxicity to the cells. Due to it's these properties it has the potential to replace the petrochemical based polymers. It is gaining popularity in various fields involving packaging, medical and coating materials. PHA has been manufactured for non-woven materials, polymer films, sutures, and pharmaceutical products used insurgery, transplantology, tissue engineering, and pharmacology [40]. In tissue engineering, the cells are grown in vitro on biodegradable polymers to construct "tissue" for implantation purposes [41].

CONCLUSION AND FUTURE OUTLOOK

This review presents the PHA as potential substitute material to some conventional plastics has drawn much attention due to the biodegradable and biocompatible properties of PHA. The potential applications of PHA in various industries and in the medical field are encouraging. But the production cost of PHA has been a major drawback. Consequently, scientists have shown immense interest in searching for new bacterial strains, creating new types of recombinant strains and tailoring various kinds of PHA to reduce the cost of production. The ongoing commercialization activities in several countries are expected to make PHA available for applications in various areas soon.

ACKNOWLEDGEMENT

The author acknowledges sincere thanks toDCRUST, Murthal, Sonipat, and Haryana, India for carrying out the research work successfully and for providing the assistance for collection of material for the review.

REFERENCES:

[1] Doi Y. *Microbial polyesters*. New York, VCH. **1990**.

[2] Findlay RH, White DC. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Applied and Environmental Microbiology*, **1983**, 71-78.

[3] Williamson DH, Wilkinson JF. The isolation and estimation of the poly-β-hydroxy-butyrate inclusions of *Bacillus* species.*Journal of General Microbiology*, **1958**, 198-209.

[4] Forsyth WGC, Hayward AC, Roberts JB. Occurrence of poly-β-hydroxybutyric acid in aerobic Gram-negative bacteria. *Nature*, **1958**, 800-801.

[5] Hassan MA, Shirai Y, Kubota A, Abdul Karim MI, Nakanishi K, Hashimoto K. Effect of oligosaccharides on glucose consumption by *Rhodobacter sphaeroides* in polyhydroxyalkanoate production from enzymatically treated crude sago starch.
Journal of Fermentation and Bioengineering, **1998**, 57-61.

[6] Hassan MA, Shirai Y, Kusubayashi N, Karim MIA, Nakanishi K, Hashimoto K. Effect of organic acid profiles during anaerobic treatment of palm oil mill effluent on the production of polyhydroxyalkanoates by *Rhodobacter sphaeroides*. *Journal of Fermentation and Bioengineering*, **1996**, 151-156.

[7] Hassan MA, Shirai Y, Kusubayashi N, Karim MIA, Nakanishi K, Hashimoto K. The production of polyhydroxyalkanoate from anaerobically treated palm oil mill effluent by *Rhodobacter sphaeroides*. *Journal of Fermentation and Bioengineering*, **1997**, 485-488.

[8] Hashimoto K, Tsuboi H, Iwasaki S, Shirai Y. Effect of pH on the production of poly-β-hydroxybutyrate by photosynthetic bacterium, *Rhodospirillum rubrum*. *Journal of Chemical Engineering of Japan*, **1993**, 56-58.

[9] Jau MH, Yew SP, Toh PSY, Chong ASC, Chu WL, Phang SM, Najimudin N, Sudesh K. Biosynthesis and mobilization of poly(3-hydroxybutyrate) [P(3HB)] by *Spirulina platensis*. *International Journal of Biological Macromolecules*, **2005**, 144-151.

[10] Jensen TE, Sicko LM. Fine structure of poly-β-hydroxybutyric acid granules in a blue-green alga, *Chlorogloea fritschii*. *Journal of Bacteriology*, **1971**, 683-686.

[11] Dawes EA, Senior PJ. The role and regulation of energy reserve polymers in microorganisms. *Advance in Microbial Physiology*, **1973**, 135-266.

[12] Macrae RM, Wilkinson JF. The influence of culture conditions on poly-β-hydroxybutyrate synthesis in *Bacillus megaterium*.*Proceedings of the Royal Physical Society of Edinburgh*, **1958**, 73-78.

[13] Macrae RM, Wilkinson JF. Poly-beta-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *Journal of General Microbiology*, **1958**, 210-222.

[14] Steinbüchel A, Valentin HE. Diversity of bacterial polyhydroxyalkanoic acids. FEMS *Microbiology Letters*, **1995**, 219-228.

[15] Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. *Progress in Polymer Science*, **2000**, 1503-1555.

[16] Wallen LL, Rohwedder WK. Poly-β-hydroxyalkanoate from activated sludge. *Environmental Science and Technology*, **1974**, 576-579.

[17] De Smet MJ, Eggink G, Witholt B, Kingma J, Wynberg H. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. *Journal of Bacteriology*, **1983**, 870-878.

[18] Witholt B, Kessler B. Perspectives of medium chain length poly (hydroxyalkanoates), a versatile set of bacterial bioplastics. *Current Opinion in Biotechnology*, **1999**, 279-285.

[19] Kunioka M, Kawaguchi Y, Doi Y. Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. *Applied Microbiology and Biotechnology*, **1989**, 569-573.

[20] Saito Y, Nakamura S, Hiramitsu M, Doi Y. Microbial synthesis and properties of poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Polymer International*, 1996, 169-174.

[21] Kellerhals MB, Kessler B, Witholt B, Tchouboukov A, Brandl H. Renewable long-chain fatty acids for production of biodegradable medium-chain-length polyhydroxyalkanoates (mcl-PHAs) at laboratory and pilot plant scales. *Macromolecules*, **2000**, 4690-4698.

[22] Lee SY, Wong HH, Choi JI, Lee SH, Lee SC, Han CS. Production of medium-chain-length polyhydroxyalkanoates by

high cell-density cultivation *Pseudomonas putida* under phosphorus limitation. *Biotechnology and Bioengineering*, **2000**, 466-470.

[23] Yu P, Chua H, Huang AL, Ho KP. Conversion of industrial food wastes by *Alcaligenes latus* into polyhydroxyalkanoates. *Applied Biochemistry and Biotechnology*, **1999**, 445-454.

[24]Omar S, Rayes A, Eqaab A, Voβ I, Steinb・hel A. Optimization of cell growth and poly (3-hydroxybutyrate) accumulation on date syrup by a *Bacillus megaterium* strain. *Biotechnology Letters*, **2001,** 1119-1123.

[25] Chee JY, Tan Y, Samian MR, Sudesh K. Isolation and characterization of a Burkholderia sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymer and the Environment*, **2010**, in press, doi: 10.1007/s10924-010-0204-1.

[26] Thakor N, Trivedi U, Patel KC. Biosynthesis of medium chain length poly (3-hydroxyalkanoates) (mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils. *Bioresource Technology*, **2005**, 1843-1850.

[27] Yu J, Stahl H. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. *Bioresource Technology*, **2008**, 8042-8048.

[28] Lee WH, Loo CY, Nomura CT, Sudesh K. Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. *Bioresource Technology*, **2008**, 6844-6851.

[29] Cavalheiro JMBT, de Almeida MCMD, Grandfils C, da Fonseca MMR. Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry*, **2009**, 509-515.

[30]Loo CY, Lee WH, Tsuge T, Doi Y, Sudesh K. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. *Biotechnology Letters*, **2005**, 1405-1410.

[31]Fonseca GG, Antonio RV. Polyhydroxyalkanoates production by recombinant *Escherichia coli* harboring the structural genes of the polyhydroxyalkanoate synthases of *Ralstonia eutropha* and *Pseudomonas aeruginosa* using low cost substrate. *Journal of Applied Sciences*, **2006**, 1745-1750.

[32] Marsudi S, Unno H, Hori K. Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology*, **2008**, 955-961.

[33] Fernadez D, Rodrauez E, Bassas M, Viňas M, Solonas AM, Llorens J, Marques AM, Manresa A. Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions. *Biochemical Engineering*, **2005**, 159-167.

[34]Simon-Colin C, Raguenes G, Crassous P, Moppert X, Guezennec J. A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei biovar. tikehau*, isolated from a "kopara" mat of French Polynesia. *International Journal of Biological Macromolecules*, **2008**, 176-181.

[35] Pantazaki AA, Papaneophytou CP, Pritsa AG, Liakopoulou-Kyriakides M, Kyriakidis DA. Production of polyhydroxyalkanoates from whey by *Thermus thermophilus* HB8. *Process Biochemistry*, **2009**, 847-853.

[36]Kshama Lakshman & Tumkur Ramachandriah Shamala : Department of food and microbiology, Central food technological research institute , Mysore 570013, India, Enhanced biosynthesis of PHA in a mutant strain of Rhizobium meliloti, **2002.**

[37]. Shake flask studies and Fed batch fermented studies in *Alcaligenes latus* & *Alcaligenes eutrophus*: by middle east technical university, Department of biological sciences, Biotechnology research unit 06531 Ankara-Turkey

[38]. Ojumu, T.V., Yu, J. and Soloman, B.O.: Biodegradation of polymer engineering material department institute, P.M.B 611, Akure, Nigeria Hawaii Natural energy institute, university of Hawaii, Honolulu HI 96822, USA Department of chemical engineering, Obafemi Awolowo university, Nigeria, **2003.**

[39]. Robert G Kranz, Karen K. Gabbert, Terry A. locke and Michael T. madigan (1997): PHA production in *Rhodobacter capsulatus*. Department of biology, Washington University, St. louis Missouri 63130& Department of microbiology, Southern Illinois university Carbondale Illnois 62901-6508

[40] Noda I. Process for recovering polyhydroxyalkanoates using air classification. United States Patent, **1998**, 849,854.

[41] Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, Vacanti JP, Mayer JE. Creation of viable pulmonary artery autografts through tissue engineering. *The Journal of Thoracic Cardiovascular Surgery*. **1998**, 536-546.

[42] Keen I, Broota P, Rintoul L, Fredericks P, Trau M, Grøndahl L. Introducing amine functionalities on a poly (3-

hydroxybutyrate-co-3- hydroxyvalerate) surface: comparing the use of ammonia plasma treatment and ethylenediamine aminolysis, Nanotechnology and Biomaterials Centre and School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, Queensland, Australia **, 2003.**