**Polyhydroxyalkanoates: characteristics, production, recent developments and applications**

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**Abstract**

Polyhydroxyalkanoates (PHAs) are biopolymesters, stored within cells as energy storage materials by various microorganisms. Due to their biocompatibility and biodegradability, PHAs have a wide range of applications in various industries such as biomedical sector including meniscus repair devices, bone plates, tendon repairing, pericardial patches, bone marrow scaffolds, tissue engineering, wound dressings, in addition to use as biofuel, drug delivery carriers and biosensors. PHAs are green plastics and they have positive social and environmental impact when compared to conventional plastics in terms of production and recycling. These bioplastics represent a renewable and sustainable resource to reduce landfill requirements without persistence or pollution. This review outlines production and characteristics of PHAs, developments in their production, and applications in various industries including nanotechnology.

**Keywords:** Polyhydroxyalkanoates; Biopolyester; Bioplastic; Nanotechnology; Polyester
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1. Introduction

The production of petroleum based plastics has a big disadvantage of generating recalcitrant non-degradable waste products (Akaraonye et al., 2010). The non-biodegradable nature of these products, due to their high molecular weight and structure recalcitrance, imparts a high environmental burden as they can remain in water bodies, soil and landfill for many years. Concerns over the harmful effects of these petrochemical derived plastics by public and environmental bodies have been increasing. This awareness prompted a global scientific drive to develop alternative green, ecofriendly and biodegradable polyesters and plastics substitutes.

The term “polyester” refers to polymers containing ester groups in their molecular chains. Microbial fermentation produces polymeric materials with the most common building blocks as shown in Fig.1. Except polyhydroxyalkanoates, which is carried out in vivo, all other polymers are polymerized in vitro by chemical reactions (Fig. 1) (Chen, 2010).

2. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are bio-polymers, synthesized by microorganisms as lipid inclusions for energy storage in granular forms within the cellular structure (Poli et al., 2011b). The French scientist Lemoigne first discovered PHA in Bacillus megaterium in the form of poly (3-hydroxybutyrate) (PHB) in 1925 (Chee et al., 2010). PHAs are natural polyesters of 3-, 4-, 5-, and 6-hydroxyalkanoic acids which are thermoplastics. To date, more than 90 genera of both Gram-positive and Gram-negative bacteria have been identified as PHAs producers under both aerobic and anaerobic conditions (Kim et al., 2007; Zinn et al., 2001). Many microorganisms can store intracellular inorganic and/or organic inclusions which are surrounded by phospholipids. For instance, if the inclusion has an iron oxide core, it is called inorganic inclusion like magnetosomes; while, if the inclusion has a polyester core, it is called organic inclusion such as PHAs (Poli et al., 2011; Rehm, 2007). In the case of PHAs, the core of polyester is surrounded by either phospholipids or proteins. Bacteria store PHAs within the cytoplasm where PHAs exist in granules ranging in size from 0.2-0.5µm.
Bacteria can be divided into two groups with regards to PHAs production. In the first group, bacteria require limitation of a nutrient such as phosphorous, nitrogen, oxygen or magnesium to accumulate PHAs and they do not accumulate PHAs during the growth phase (Muhammadi et al., 2015). The second group accumulates PHAs during the growth phase and do not require any nutrient limitation (Muhammadi et al., 2015). For example, bacteria *Ralstonia eutropha*, *Pseudomonas oleovorans* and *Pseudomonas putida* belong to the first group, while, recombinant *Escherichia coli* belongs to the second one (Nitschke et al., 2011).

### 2.1. Structure of PHAs

About 150 different congeners of PHAs have been reported (Zhang et al., 2006). The general structure of PHAs is shown in Fig. 2. If the group is $R=\text{CH}_3$, the resultant polymer is called polyhydroxybutyrate or polyhydroxybutyric acid, while if $R=\text{C}_3\text{H}_7$, the polymer is called polyhydroxyoctanoate (PHO) and so on.

### 2.2. Classification of PHAs

PHAs are classified into three classes of short, medium or long chain (scl, mcl and lcl) respectively, according to the number of carbons in the side chains (Kunasundari and Sudesh, 2011). The scl-PHAs have less than 5 carbon atoms, while, mcl-PHAs have 5-14 carbon atoms and lcl-PHAs have more than 14 carbon atoms but are uncommon and less studied. The congeners of 3-hydroxyvalerate and 3-hydroxybutyrate are examples of scl-PHAs, while 3-hydroxydecanoate, 3-octanoate and 3-hydroxyhexanoate are of mcl-PHAs. Fig. 3 shows the general structure of PHAs with their classification. The mcl-PHAs were first observed in *P. oleovorans* in 1983 (Rai et al., 2011).

### 2.3. Properties of PHAs

Due to the structural variations in monomers constituting PHAs, they differ in properties and chemical composition as homo or co-polymers. PHB is comparable to polypropylene and shows good resistance to moisture and acquire excellent barrier properties to gases. PHAs are insoluble in water, have good resistance to hydrolytic attack, resistant to UV, sink in water which facilitates anaerobic biodegradation in sediments, being biocompatible and biodegradable (i.e. undergoes degradation in soils) and behave as piezoelectric materials (Bugnicourt et al., 2014). PHAs also have chiral molecules and the degradation of PHAs depends mainly on their type and
composition (Boyandin et al., 2013). Therefore, the biodegradation of PHAs is affected by the type and composition of the polymer, environmental conditions and the type of microorganisms (different microorganisms produce different PHA-depolymerases to degrade PHAs) (Masood et al., 2014).

PHAs are soluble in chloroform and other chlorinated solvents. Their glass transition temperature varies from -50 to 4°C, melting temperature from 40-180°C (Padermshoke et al., 2005). Thermo-degradation temperature, tensile strength, Young’s modulus, water vapor and oxygen transmission rate vary according to the type of polymer produced and the composition of monomeric unit (Bugnicourt et al., 2014).

2.4. Microorganisms producing PHAs

Both prokaryotic and eukaryotic type of microorganisms can produce different types of PHAs. PHAs have also been reported in blood and tissues of human and animals (Rehm, 2009), and are used to control of seizure, metabolic disease and increasing cardiac efficiency. Different bacteria produce different type of PHAs. Fluorescent Pseudomonas strains, for example, are well known to accumulate mcl-PHAs as they have mcl-PHA synthases for the synthesis of PHAs with 6-14 carbon atoms (Kim et al., 2000).

2.5. Carbon sources on PHAs composition

The composition of the PHAs produced by different bacterial strain varies depending on the type of carbon source available. Such property is due to the physiological and biochemical characteristics of the microorganism for biochemical synthesis as well as the adopted production conditions (Volova, 2004). Pseudomonas putida had a tendency to synthesize PHAs by incorporating different functional groups like phenyl, phenoxy, olefin, halogens, alkyls and esters when grown on substrates containing the corresponding chemical structures (Kim et al., 2000). P. putida is also useful for the production of PHAs with cyanophenoxy, nitrophenoxy, methylphenoxy, phenoxy and triple carbon-carbon bonds. In a study, 36 different carbon sources were used to synthesize a range of PHAs through investigation on P. putida KCTC2407 for PHAs production (Kim et al., 2000). P. putida was unable to use short-chain carboxylic acids containing bromine, ethoxy, cyclohexyl, methoxy, phenoxy and olefin functional groups as carbon sources.
The cost of PHAs synthesis is usually a major factor that limits its industrial applications. Different carbon sources have been reported to make the production process cost effective. *Pseudomonas* species strain DR2 was used for the production of PHAs using glucose, citrate, glycerol, acetate, butyrate, palmitate, corn oil and waste fried oil as sole carbon source; among them corn oil showed maximum PHAs (37.3%, w/w) of dry weight (CDW) followed by waste fried oil at 23.5% (w/w) of CDW when cultivated at 30°C for 72h (Song et al., 2008). Recently, a gamma ray mutant strain of *P. aeruginosa* was reported to produce 50.3% (w/w) PHA with soybean oil (Abid et al., 2016), and 40.7% (w/w) with *n*-hexadecane (Raza et al., 2016). Therefore, choice of carbon source is a crucial factor to control the yield of PHAs.

**2.6. C/N ratio on PHAs production**

The effect of carbon/nitrogen (C/N) ratio on cell growth and PHAs accumulation had been studied (Ahn et al., 2015; Cui et al., 2017; Supono et al., 2013). Generally, an increase of C/N ratio promotes PHAs accumulation while the reverse effect was observed for cell growth. Basaket al. (2011) reported that nitrogen limitation induces better accumulation of PHAs using activated sludge waste as growth substrate, while, maximum production was 43.3% (w/w) of CDW. In another study using *P. nitroreducens*, the C/N ratio had significant influence on PHAs accumulation and composition (Yao et al., 1999). An increased C/N ratio of the culture medium led to reduction of 3-hydroxybutyrate content of PHAs from 100 to about 7% (w/w), while, PHAs accumulation increased from 0.5 to 33.5% even though a negative impact on the cell growth was observed (Yao et al., 1999). During fermentation, the strategy is to keep carbon source in excess for PHAs production (Ebnesajjad, 2012; Preusting et al., 1993; Sudesh, 2012; Montie, 2013). On the other hand, PHAs accumulation up to 70% (w/w) of CDW was reported without nutrient limitation using *P. putida* KT2440 (Sun et al., 2007). Table 1 gives an overview of some literature on PHAs production.

**3. Cost associated with PHAs production**

Although microbial plastics have a distinct advantage over conventional petroleum-based plastics, the main drawback is the high cost associated with fermentative production (Chaudhry et al., 2011). The major factors affecting the cost of production include the type of carbon source, running cost of fermentation, process productivity, yields on the selected carbon sources and downstream processing (Lee and Na, 2013). The PHAs contents in the cell biomass are very
important for economical extraction process, also the selection of a hyper productive microorganism is equally important for a cost effective production. For example, the average USD/kg cost of PHB production (100,000 ton/year) was found to be 2.6, 5.37 and 6.69 from *Alcaligenes latus*, *E.coli* and *Methylobacterium organophilum*, respectively (Choi and Lee, 1999).

### 3.1. Lowering the cost of PHAs production

Despite the high-profile properties of PHAs, they are still not comparable with the petroleum-based plastics due to the cost associated with their production at 5-10 times higher than conventional petrochemical based plastic such as polyethylene. The cost of carbon substrate is the main factor amounting to 50% of the overall production cost (Kim, 2000). Worldwide research interest therefore is focusing on reducing the cost of production using different waste materials as carbon source (Alvi, 2014).

#### 3.1.1. Whey, wheat and rice bran as carbon source

Whey is the by-product from cheese and casein industry representing up to 90% of the volume of processed milk. Half of this whey is converted into usable products for human and animals and the rest usually is discarded into the environment. Attempts to use whey as carbon source for bacterial growth as a cheap substrate for PHAs production strategy were investigated. PHA yield of 1.27 g/l with a biomass yield of 5.0 g/l by using *P. hydrogenovora* was reported (Koller et al., 2008).

Wheat is cultivated worldwide in large amounts. Bran is the outer layer of the wheat grain which is hard and consists of pericarp and aleurone, and is one of the integral parts of the grain. It contains proteins, carbohydrates and other minerals, and its disposal can be problematic. Trials using wheat bran waste as carbon source for growth and PHAs production were carried out by growing *Halomonas boliviensis* LC1 resulting in biomass production of 3.19 g/l and PHB production of 1.08 g/l (Van-Thuoc et al., 2008). On the other hand, rice is another major crop cultivated worldwide. A high biomass content up to 140 g/l and PHAs accumulation of 55.6% (w/w) of CDW were reported using rice bran along with corn starch using *Haloferax mediterranei* (Huang et al., 2006). Thus, the use of these wastes can be a credible alternative to produce PHAs and to reduce the problems of their disposal into the environment.
3.1.2. Starch as carbon source

Starch is a potential cheap material available in the market, however, it has quite complex nature which limits the abilities of many bacterial strains to hydrolyze it and produce α-amylase. Hence, external source of this enzyme is required by such microorganisms to hydrolyze starch and use as carbon source. The bacterium *Haloferax mediterranei* along with enzymatic extruded starch gave biomass yield of 1.14 g/l with PHAs accumulation of 43% (w/w) of CDW (Chen et al., 2006). Using potato starch as a sole carbon source in high cell density fed-batch culture conditions for bacterial strain *Ralstonia eutropha* NCIM 5149, a total cell biomass of 179 g/l with PHAs content of up to 55% (w/w) of CDW was reported (Haas et al., 2014).

3.1.3. Sugar-cane molasses as carbon source

Sulphured and un-sulphured molasses are two types of molasses which are viscous by-products of sugar cane extraction with or without the use of sulphur dioxide as stabilizer. Sugar cane molasses contains sucrose, glucose, fructose in addition to iron, magnesium, calcium, potassium and vitamins including B7 all of which are suitable for bacterial cells growth (Shasaltaneh et al., 2013). Upto 6.0 g/l of PHAs were reported when sugar cane molasses were used as a carbon source (Santimano et al., 2009). Sugar-cane molasses has been utilized by *Pseudomonas* species to produce 1.67 g/l biomass and 1.45 g/l rhamnolipids (Raza et al., 2007). In a study of PHAs accumulation using sugar cane molasses, lower pH during fermentation favored butyric acid and valeric acid production, while, at higher pH, acetic acid and propionic acid were the main products; however, high ammonia concentration had a negative impact on PHAs accumulation (Albuquerque et al., 2007). *P. fluorescens* A2a5 was grown on cheap sugar cane liquor medium as a carbon source and produced PHAs at upto 70% (w/w) of CDW, thus significantly lowering the cost of PHAs production (Jiang et al., 2008).

3.1.4. Waste vegetable oils and plant oils as carbon sources

Fast food industries produce large amounts of waste frying vegetable oils which need proper disposal as they have high chemical and biological oxygen demands. Waste frying oils are suitable substrates for the production of rhamnolipids as well as PHAs (Haba et al., 2007; Hori et al., 2002; Raza et al., 2006). Many strains of bacteria are reported to produce PHAs from these waste frying oils; however, yields are usually low with plant oils (Akaraonye et al., 2010). Haba et al.(2007) reported the production of up to 7.6 g/l of PHAs and 10 g/l of rhamnolipids,
simultaneously using *P. aeruginosa* 47T2. During characterization of PHAs produced by waste frying oils, up to seven congeners had been identified. The most abundant monomers in the composition were C\textsubscript{10:0} and C\textsubscript{12:0} representing 47.8 and 15.3% (w/w) of the PHAs, respectively, while unsaturated monomers were 26.2% (w/w) of the PHAs (Haba et al., 2007). To successfully transfer the carbon source to the bacterial cells, the role of lipases and rhamnolipids is very important. Lipases hydrolyze the fats, while, rhamnolipids reduces the surface tension and promotes the emulsification of oil in water making easier access of bacterial cells to the carbon source (Abid et al., 2016).

*P. aeruginosa* 42A2 showed different PHA yields on different substrates; 66.1% from waste free fatty acids, 29.4% from waste frying oils and 16.8% from glucose and 54.6% from oleic acid (Fernandez et al., 2005). The nutrient limitation selection is crucial for better PHAs yield. For example, *P. aeruginosa* 47T2 when grown on waste oils showed varied PHAs yield with variation in temperature of incubation, carbon source, phosphorous and nitrogen limitation (Haba et al., 2007). Lower levels of PHAs were observed at higher temperatures; 28.2% at 37\textdegree C and 2.2% (w/w) of CDW at 42\textdegree C (Haba et al., 2007). *P. aeruginosa* IFO3924 was studied for PHAs and rhamnolipids production, simultaneously, using oleic acid, glycerol and palm oil. The C\textsubscript{8} and C\textsubscript{10} were the major congeners of the produced PHAs under both batch flask and bioreactor conditions using a basal salt medium with 5-15g/l palm oil at 28\textdegree C (Marsudi et al., 2008). It was noted that the product yield increased upon increasing the concentration of palm oil from 5 to 7.5 g/l and decreased at higher concentration of 10 and 15 g/l (Marsudi et al., 2008). During downstream processing of PHAs from cells, the attached oil with cells and PHAs are typically removed by solvent extraction and separation (Raza et al., 2016). Recently, 6 bacterial strains were tested for PHAs production using 4 different carbon sources including; waste frying oils, diesel, canola oil and glucose. Among these four carbon sources, maximum PHAs accumulation of 53.2% (w/w) was achieved using *P. aeruginosa* (KF270353) when grown on waste frying oils, while, 37.8 and 34.4 % (w/w) when grown on glucose and canola oils, respectively (Tufail et al., 2017).

### 3.1.5. Wastewater for PHAs production

Wastewater could be treated to remove impurities such as gases and organic solvents, and can be used to produce PHAs by mixed culture method. PHA accumulation of 58% (w/w) of CDW had been reported when growing *Azotobacter vinelandii* UWD strain on swine waste-water (Ryu et
al., 2008). PHA yield of 43% (w/w) of CDW had been reported when acetate was used as additional carbon source using paper and pulp wastewater (Yan et al., 2006). Textile waste-water had also successfully been utilized for the production of PHAs as well as for the bio-degradation of dyes (Tamboli et al., 2010).

3.2. Limitations and advantages of PHAs production using wastes

Most of the times it had been beneficial to use waste products to develop new products solving both economic and environmental issues. The utilization of waste materials for the synthesis of high-class materials like PHAs has led to cost reduction. Life cycle assessment investigations showed that the production of PHAs from whey waste is economically preferable to converting whey waste into powder form (Koller et al., 2013). Different waste products results in different PHA yield and cell biomass. PHA contents as low as 8% to as high as 89.10 % (g/g of CDW) has been reported in literature from different waste materials like whey, starch, tallow, spent coffee grounds, wheat and rice straw, fermented mash, spent wash, molasses, Sugarcane bagasse and vinasse, bean curd waste and waste frying oils (Bhattacharyya et al., 2012; Cesario et al., 2014; Chaudhry et al., 2011; Kim, 2000; Koller et al., 2005; Kumalaningsih et al., 2011; Lopes et al., 2014; Obruca et al., 2014; Pais et al., 2016; Sindhu et al., 2013; Taniguchi et al., 2003; Vastano et al., 2015). Although, the utilization of waste is advantageous, yet the presence of impurities and the variation in waste compositions each time results in varying final PHAs production yields. Such varying output from these processes would require prior PHAs optimization at laboratory scale before bulk trials whenever a new waste stock is used resulting in additional cost and uncertainty in output (i.e., yield). For example, virgin oil provided better PHAs yield as compared to waste frying oils under similar conditions which was attributed, by the researchers, to the presence of impurities in waste oil (Du et al., 2012).

Although, the interest in using waste products for the biosynthesis of PHAs is associated with substantial benefits in reducing the waste disposal problem, yet the finished products could not be used in medical applications where high purity products with non-toxic nature are of utmost considerations. In addition, PHAs produced from wastes could have viral, plasmid, bacterial or genetic contaminations which prevent their usage for medical grade applications. Various impurities affiliated with endotoxins, proteins, lipids, antifoam agents, DNA and hypochlorite have been reported (Koller et al., 2013). These impurities require different washing procedures
post recovery of PHAs resulting in cost increases in the products (Boynton et al., 1999; Holmes et al., 1980; Volova et al., 2003). Such cost increases associated with improved purity are often not an issue when these products are to be used in medical applications.

4. Extraction of PHAs from bacterial cells

As PHAs are stored within the cells, the methods to recover biopolymer are complex and costly. There are various methods described in the literature for the extraction and downstream processing of PHAs, each method is associated with some advantages and disadvantages. Widely used methods for the recovery are mentioned below.

4.1. Solvent extraction

Solvent extraction is the most commonly used technique for the extraction of PHAs due to its simplicity and ease of operation. It involves the following steps; a pre-treatment to rupture the cells so that the PHA granules become accessible and then, making these granules soluble in a suitable solvent followed by precipitation with a non-solvent. Chlorinated solvents are used for the solubilization of PHAs from ruptured cell biomass including chloroform, 1,2-dichloroethane, acetone, 1,2-propylene carbonate and ethylene carbonate. Acetone is normally preferred for mcl-PHAs. Methanol or ethanol in a chilled form is used as non-solvent for precipitation. Solvent extraction is utilized when high purity is required as it does not degrade the polymer and also removes the endotoxins of the cells (Jacquel et al., 2008). Moreover, 1,2-propylene carbonate is the preferred solvent due to its lower toxic nature as compared to others.

In a study mainly involving polyhydroxyoctanoate extraction using different solvents such as tetrahydrofuran (THF), 2-propanol, n-hexane, ethyl acetate, acetone and methylene chloride; the most efficient solvents was methylene chloride at room temperature giving about 86% (w/w) recovery (Furrer et al., 2007). Acetone and ethyl acetate showed impurities less than 10% and subsequent precipitation with methanol increased purity of polymer extracted to nearly 100%. Except for 2-propanol, all solvents used resulted in an efficient recovery of low endotoxin containing mcl-PHAs from bacterial biomass with up to 100% purity. At around 50°C, n-hexane showed good extraction but lowering the temperature to 40°C, the recovery was decreased but the product had less endotoxin (Furrer et al., 2007).
4.2. Floatation method

The floatation method is the modification of solvent extraction in which a solvent is employed for the extraction of PHAs followed by the self-floatation of the cell debris. The cells are mixed with chloroform at 30°C for 72 h and then, left overnight for the self-floatation of the debris to occur at room temperature. This method reported high purities up to 98% with recovery efficiency of 85% (w/w) for PHAs (Ibrahim and Steinbüchel, 2009). This method has also other advantageous including reduction of wastage of the extracted polymer and ability to use green/eco-friendly solvents.

4.3. Digestion method

Digestion methods are alternatives to solvent extraction. They involve a digestion mechanism to release PHAs from the cells. The digestion can be chemical or enzymatic. Both these techniques digest non-PHAs cellular mass. In the chemical method, typically sodium hypochlorite or surfactants are applied to facilitate the recovery of PHAs from the cell. Triton X-100, palmitoyl carnitine, sodium dodecyl sulfate and betaine have all been used with sodium hypochlorite for chemical digestion. Digestion with only sodium hypochlorite, may lead to polymer degradation up to 50%. Both surfactants and sodium hypochlorite when used separately gave poor performance; hence, the combinations of both were studied for better performance. Solvents like propylene carbonate, dichloroethane, methylene chloride and chloroform could be used to extract pure PHAs from the cells; these chemicals however are toxic. Enzymatic digestion contains many steps consisting of heat treatment, enzymatic hydrolysis and surfactant washing. Enzymes are known to be target specific and hence could achieve good recovery of PHAs. A high recovery of PHB (5.6 g/l) has been reported using digestion method as compared to others (1.1 g/l by dispersion method and 0.63 /l by chloroform extraction) (Sayyed et al., 2009)

4.4. Supercritical fluids

Supercritical fluids are the latest technology advancements used now-a-days due to their low toxic nature and cost. Supercritical carbon dioxide, due to its moderate temperature and pressure is dominating the industry for recovering the PHAs from bacterial cells. The speed of percolation of supercritical fluids is high due to their less viscosity and almost no surface tension, which
results better diffusion than liquids for extraction purposes. Temperature and operating pressure are key parameters as high temperature and pressure can modify the cell membrane making it more difficult to extract the polymer from the cells. Upto 89% recovery of PHA of CDW has been reported using this technique with *Ralstonia eutropha* cells. Apart from carbon dioxide gas, ammonia and methanol had also been used for supercritical fluid method to recover PHAs. Based on CDW, 42.4% (w/w) yield of mcl-PHAs from the lyophilized cells of *Pseudomonas resinovorans* grown on tallow and lard had been reported using supercritical carbon dioxide and chloroform extraction. Darani et al. (Khosravi-darani et al., 2003) reported that both, temperature and pressure, significantly affected the solubility of PHB in supercritical carbon dioxide. Even after solvent extraction, impurities like endotoxins, which are heat resistant, can go along the final product and may cause inflammatory reaction when used *in vivo* (Koller et al., 2013). Super critical solvent extraction as described earlier is an effective extraction process to produce impurities-free PHAs for medical applications. Comparing with conventional extraction process, super critical solvent extraction method provide 100% purity of recovered PHAs and products having about 150 times less impurities (Williams et al., 2005).

### 4.5. Aqueous two-phase extraction

Aqueous two-phase extraction (ATPE) is environmental friendly approach as compared to solvent extraction systems since it is comprised of two unique phases i.e., water and non-volatile phase. The ATPE system utilized water to isolate, purification and recovery of PHAs. The ATPE is known to remove undesired impurities from the product (Kepka et al., 2003). Upto 51% PHAs recovery has been reported from *Bacillus flexus* cells using ATPE with 12% (w/v) polyethylene glycol and 9.7% (w/v) potassium phosphate (Divyashree et al., 2009). The PHA was transferred to the polyethylene glycol layer while the cell debris was settled down in the bottom layer. Thus, it can be said that ATPE is a non-solvent yet effective method to isolate the PHAs from bacterial cells.

### 5. Downstream process on PHAs production cost

The final cost of PHAs production, as discussed earlier, depends on many factors including downstream processing. Various methods have been reported with different product yield, energy and time requirement, and product purity (Koller et al., 2014). Therefore, the selection from carbon source to microorganism as well as downstream processing is crucial. For example,
if a medical product is to be fabricated from the extracted PHAs, it requires high purity and cost
is less decision determining process. On the other hand, if the product must be used for single
time use disposable item, then, cost of downstream rather than purity is the key parameter.
Thermosetting ATPE uses thermo-separating polymers like ethylene oxide or polyethylene oxide
which separates into two layers upon heating and providing easy separation of required end-
products. Recently, the economy of PHAs production and downstream cost had been analyzed
and it was reported that an economical cost of 5.77 USD/kg of PHA was achieved using ATPE
(Kit et al., 2017). Therefore, the use of ATPE can be considered as environmental friendly down
streaming process for isolation and recovery of PHAs (Yau et al., 2015). Thus, ATPE has
become an economical and industrially viable tool for purification and purification (Zijlstra et
al., 1998).

6. Synthetic biology, genetic and metabolic engineering strategies for PHAs

Current advancements in synthetic biology, genetic and metabolic engineering have intensified
the usage and application of different interdisciplinary technologies in biological cells to
manipulate the biochemical processes. Synthetic biology has been utilized to deliver various
technical products using bacteria and yeasts (Keasling, 2008). Combination of metabolic
ingeniering and synthetic biology has been reported for the improvement in bio-catalytic activity
of PHA synthase, thus increasing the amount of biosynthesized PHAs (Matsumoto et al., 2006).

Genetic engineering mainly deals with biochemistry, genetics and biology while synthetic
biology deals with mathematics and systems biology (Hu and Dhar, 2015). The use of low
molecular mass protein like “Phasins” for the secretion of PHAs has been reported to decrease
the cost associated with the extraction of PHAs from the bacterial cells using synthetic biological
engineering as there remains no need to disturb the cells for extraction (Rahman et al., 2013).
The Phasins target the PHB and reduces the size which helps in secretion of Phasins bound PHB.
Parental strain of Herbaspirillum seropedicae SmR1 showed up to 50% reduction in PHB
accumulation when PhaP1 protein was deleted (Alves et al., 2016). Metabolic engineered
methanol utilizing bacteria Methylobacterium extorquens was capable to produce functionalized
PHB when cloned with the genes of P. fluorescens GK13 (Hofer et al., 2010).

The yeast Yarrowia lipolytica, does not have the capability to produce PHAs naturally.
Genetically engineered Y. lipolytica has been reported to produce 1.11 g/l PHAs when grown on
oleic acid (Gao et al., 2015) and enhanced PHAs production (up to 7.3% w/w of CDM) were observed by modifying β-oxidation protein (Haddouche et al., 2011). By reversing the β-oxidation cycle *E. coli* produced up to 6.62% (w/w) mcl-PHAs (Zhuang et al., 2014). Genetically modified tobacco plant has also been reported to accumulate 4.8 mg/g mcl-PHAs (Wang et al., 2005).

Gram-negative bacteria are used widely for the biosynthesis of PHAs. While, Gram-positive species is known to be non-endotoxin producers but lack the PHAs producing genes. Therefore, if PHAs producing genes could be inserted in them using synthetic biology and genetic engineering, the output product can be pure without endotoxin associated with them. Currently, scientists are trying to achieve the said production and a few reports have confirmed the practicality of the concept (Song et al., 2012; Valappil et al., 2006). In another report, gram-positive bacteria has been reported to dominate in the production of PHAs from waste products (Bhuwal et al., 2013). In depth analysis of *Bacillus* spp. proved that this gram-positive species can produce homo and co-polymer of PHAs from simple carbon source like sugar to waste streams and 70-80% PHAs accumulation of CDW with no endotoxin (Sonakya et al., 2001). Though, a process having upto 80% PHAs accumulation can be adopted in industry, it can further be optimized by genetic engineering to increase the PHAs accumulation levels to have maximum medical grade output. Moreover, other gram-positive bacteria can be screened and modified for better yield.

### 7. Applications of PHAs

Due to their biocompatibility, biodegradability and green credentials, PHAs are being extensively used in many fields (Chen, 2010). The PHAs polymers can be utilized as biofuels through the methyl esterification technique, medical and pharmaceuticals, and have many other applications including the following:

#### 7.1. Tissue engineering

PHB and 3-hydroxyvularate (PHBV) can be used as matrices for the *in vitro* proliferous of different human cells. The different cells like endothelium cells, isolated hepatocytes and
fibroblasts show similar adhesion to PHB and PHBV when used for matrices. So, these materials may be useful for such applications (Sevastianov et al., 2003).

PHAs were tested for bone tissue engineering and showed no chronic inflammation even after one year of exposure/use and bone formation occurred rapidly close to PHB/hydroxyapatite composite materials used for bone tissues regeneration. The addition of hydroxyapatite (HA) is carried out in PHB material to make it hard so that it may be used for the hard tissue replacement purposes (Ni and Wang, 2002). The PHB/HA composites when mechanically tested for compression strength, showed 62 MPa strength which is several times higher than the original human bones, thus making these composites ideal for fixing fracture.

PHB has also been reported for heart valve tissue engineering. Extensive research activities are in progress at present to use such PHAs polymers to construct biodegradable scaffoldings to use in the construction and engineering of living tissue heart valves for use as replacement for defective or diseased valves (Hong et al., 2009). The PHBV microspheres loaded poly (L-lactic-co-glycolic acid) (PLGA) scaffold has been reported to have more than 80% porosity (Huang et al., 2010). The compressive strength of PHBV-PLGA scaffold increased as the amount of PHBV microspheres was increased and the scaffold was successfully used in in vitro bone tissue engineering (Huang et al., 2010). The PHBV microspheres have been reported to support primary neurons when used in tissue engineering of neurons as scaffolds (Chen and Tong, 2012).

### 7.2. Bio-implant patches

Due to the biocompatible nature, low inflammatory response, biodegradability and use in tissue regeneration PHAs can be used as bio-implant patches in the human body. Local resection and closure by suture of the transmural defects in the gastrointestinal tract are often carried out as treatments. Such closure can be carried out using bio-implanted patches made of polymers such as PHB (Chen and Wu, 2005). The PHB based orthopedic implants have been reported to be used as implants in cats (Alves et al., 2011) while PHB and HA based composites were used and reported to be compatible for bone replacement in rabbits (Reis et al., 2010).

### 7.3. Drug delivery

There is vast interest in using PHAs as drug delivery carriers due to their biodegradability and biocompatibility (Pouton and Akhtar, 1996). Micro and nanospheres of PHA are utilized as
polymers that can act as outer shell in which drugs are incorporated and released when the polymer coating starts to degrade. PHAs have also been tried on many animals as drug or vaccine carriers in mice, sheep, dog, rabbit, cattle and even on human for the treatment of gingivitis (Valappil et al., 2006). Sulbactam-cefoperazone drug loaded rods of PHBV have been used to treat chronic and implant osteomyelitis. PHB has been reported for transdermal drug delivery along with polyamidoamine dendrimer. The addition of dendrimer increased the transdermal permeability of tamsulosin drug (Gursel et al., 2002).

7.4. Surgical applications

PHB tube had been used to recover damage of ureter in 1960s. The PHAs can be used for repairing damaged nerves as they are piezoelectric (Bugnicourt et al., 2014). The PHB had been used as an alternative to the conventional nerve graft and showed promising results in rats regarding nerve regeneration (Hazari et al., 1999). PHAs are also used for wound dressings and scaffolds (Volova, 2004).

8. Applications of PHAs in nanotechnology

Nanotechnology is one of the most expanding fields of science that is serving a broad spectrum of application areas. In medical science, it has particularly opened new horizons due to control of matter size at such a small scale. PHAs have found applications in the vast fields of nanotechnology due to their compatibility and uniform chirality and use as starting chemicals for many other end products (Chen, 2010). They can be used as blends with other polymers for medical applications due to high biocompatibility, as drug carriers and scaffold materials in tissue engineering.

PHAs can adhere better to lignocellulose fibers as compared to the conventional polyolefins so they can be used for the manufacturing of bio-composites to be used in nanotechnology and drug delivery (Abid et al., 2016; Pollet and Averous, 2011). Nanobiotechnology applications such as the development of protein chips using PHAs, for selective immobilization of protein are advancing for specific detection of hepatitis B virus (Rehm, 2009). PHAs are also being used for thin film/ nanogels formation for various applications (Ma et al., 2016). Solid or particulate dispersion with size range of 10-100 nm are called nanoparticles in which drugs can be entrapped, dissolved or adhered to the surface. The nanoparticles are being preferred over microparticles in medical and pharmaceutical fields due to faster intracellular uptake and fast
movement to their target (Mohanraj and Chen, 2006). The term polymeric nanoparticles is
specially given to nanospheres and nanocapsules where, nanospheres have a solid mass; while,
nanocapsules act like reservoirs of vascular system in which desired compounds are entrapped
(Rao and Geckeler, 2011). These colloidal nanoparticles are easy to apply as eye drops in liquid
form with minimum discomfort in most other forms of drugs (Zhou et al., 2013).

There are various methods for the preparation of polymeric nanoparticles, solvent evaporation
was the first one developed (Nagavarma et al., 2012). Emulsion solvent evaporation technique
had been reported to produce nanocapsules of polymers (Mora-Huertas et al., 2010; Pisani et al.,
2008). PHAs have found enormous applications in medical field including use in wound
management, vascular system, drug delivery, tissue engineering, orthopedic items and ultrasonic
imaging and so on. PHB is one of the PHAs mostly used to form of nanocapsules from
emulsification/solvent evaporation (Reis et al., 2006). Double water/oil emulsion solvent
evaporation has been reported for microcapsule preparation of pure PHB and co-polymers of
PHB with other polymers (Murueva et al., 2013).

In the last few decades, biodegradable polymers have attracted the controlled drug delivery
applications as carriers in the form of nanoparticles, nanocapsules, microparticles, microcapsules
and micro/nanospheres (Heller, 1984). PHB nanoparticles of 55 nm average diameter had been
reported produced through dialysis method of nanofabrication and when drug loaded PHB
nanoparticles were formed, it was noted that drug loading did not affect the average diameter of
the nanoparticles (Errico et al., 2009). PHB is a is hydrophobic in nature. The intestinal uptake of
hydrophobic polymers when used as drug delivery is higher than the hydrophilic ones (Zohri,
2009). For oral drug delivery, many polymers have been developed. These polymers protect the
encapsulated drug, vaccine, protein and peptides. These nano-drug carriers when administrated,
effectively distribute the drug in a uniform manner than single unit administration and it also
reduces local irritation (des Rieux et al., 2006). PHB has been demonstrated in a targeted drug
delivery system in which PhaP (a PHB binding protein) was used to bind with the polymer
through strong hydrophobic interaction fused with targeted cell ligands. Under fluorescent
microscopy, the PHB particles were visible in the targeted cells of liver and tumors due to
specific targeting effect (Yao et al., 2008).
PHB has found enormous applications in vivo. They can be used as adhesion barriers films, for better cell adhesion films as matrices for proliferous cells and for growing retinal pigment epithelium cells (Chen and Wu, 2005).

PHA nanofiber scaffolds and PHA films have been investigated for the growth of neural stem cells as replacement of natural extracellular matrix (Xu et al., 2010). Interestingly, the cells grew on this material like natural extracellular matrix. The nanofiber scaffolds of PHA showed better adhesion of cells and good viability of neural stem cells. As a porous structure is desirable for implantation, thus the PHAs proved to be a good choice to repair the damages of spinal cord injuries (Xu et al., 2010). Similarly, nanofibers based peptide modified PHA scaffold showed more adhesion to human Schwann cells. The proliferation and metabolic activity of these cells also increased, thus it had a great impact in tissue engineering (Masaeli et al., 2014).

PHAs have also been tried on many animals as drug or vaccine carriers in mice, sheep, dog, rabbit, cattle and even on human for the treatment of gingivitis (Valappil et al., 2006). Float mediated nanoparticles of PHAs had been studied for the drug delivery specific targeting (Zhang et al., 2010). PHAs have been reported for the manufacturing of nanocomposites with carbon nanotubes, where the addition of carbon nanotubes increased the crystallization of PHA, thus as a result, the crystallization temperature of nanocomposites increased along with the improvement in Young’s modulus and compression strength (Sengupta et al., 2007). Another study used PHAs with carboxyl multi wall carbon nanotubes for nanocomposites where carboxyl multi wall carbon nanotubes increased the crystallite size without affecting the structure of PHA (Valentini et al., 2014).

PHAs in the form of nano-beads have also been reported for the immobilization of proteins (Rehm, 2003). Production of these functionalized smart beads of PHAs by protein has opened new horizons in biomedical applications and a new development in this technology is the endotoxin free nano-beads of PHAs from Gram-positive bacteria (Dinjaski and Prieto, 2015). Among all the applications of PHAs, drug delivery is mostly economically viable and with a range of interesting practical sizes suitable for delivering various drugs and vaccines, reducing some of the drawbacks of conventional therapeutic treatments (Shrivastav et al., 2013). Nanoparticles based nanocomposite scaffold of PHAs and bio-glass has also been reported for use in bone related uses as they possessed less roughness and less agglomeration which could
improve cell attachment to the scaffold (Hajiali et al., 2010). Nanoparticle based PI3K inhibitor system has been reported for the reduction in proliferation of human cancer cells. The developed system was found successful but PHB showed better performance when compared to other PHAs as nano drug carriers for inhibition of cancer (Lu et al., 2011).

9. Conclusions and future perspective

PHAs are very competitive candidates to replace petrochemical plastics in selective applications due to their biodegradability. They are green materials and are used extensively in medical field. The high production cost as compared to conventional petroleum-based plastics still remains a big obstacle for PHAs growth and expansion. In the biosynthesis of PHAs, a mixture of more than one monomer is usually produced in the products rather than a single type of PHA. Major research and development is still needed to reduce the cost of synthesis and obtain effective recovery and downstream processing methods of PHAs to enhance their commercialization.

Genetically modified bacteria are one of the areas to enhance accumulation of more PHAs within the cell mass and for a single selective major structure of synthesized PHAs instead of a mixture of copolymers (normally produced polymer is a mixture of different monomers). The downstream processing cost associated with PHAs extraction should also be reduced using more cost-effective innovative methods. The choice and cost associated with the down streaming depends mainly upon the end-use of the product. These biodegradable polymers have received increasing attention, the medical related field for the development of high purity from economical extraction and recovery methods. Synthetic biology and genetic engineering can be extended to the Gram-negative bacteria to have toxin free end products with high yields.

Further reduction of production cost can open new horizons of applications in medical sciences and the life style of community can be enhanced. The current cost of PHAs production and recovery with ATPE is about 5.77 USD/kg (Kit et al., 2017), which should be reduced further to compete with conventional petrochemical competitors. PHAs can also transform the pharmaceutical technologies and future protein immobilization of PHAs may also offer other applications. These biocompatible and biodegradable polymers are the strongest candidates to replace the conventional petrochemical based plastics in the future.
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Figure 1: The most common molecular building blocks of bio-polymers produced naturally by bacterial fermentation
Figure 2: General structure of polyhydroxyalkanoates with some modifications as reported in Ojumu and coworkers (Ojumu et al., 2004), Where if n=1, R= methyl: poly (-3-hydroxybutyrate); R= hydrogen: poly (-3-hydroxypropionate); R= propyl: poly (-3-hydroxyhexanoate); R= nonyl: poly (-3-hydroxydodecanoate); R= ethyl: poly (-3-hydroxyvalerate); R= pentyl: poly (-3-hydroxyoctanoate); If n=2, R= hydrogen: poly (-3-hydroxybutyrate); If n=3, R= hydrogen: poly (-5-hydroxyvalerate).
Figure 3: Structure of PHAs with respect to classification, where 3HB = 3-hydroxybutyrate, 3HV = 3-hydroxyvalerate, 3HHx = 3-hydroxyhexanoate, 3HO = 3-hydroxyoctanoate, 3HD = 3-hydroxydecanoate and 3HDD = 3-hydroxydodecanoate.