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Advances In Phas Production

Rana Amache, Artun Sukan, Maryam Safari, Ipsita Roy, Tajalli Keshavarz*

Department of Molecular and Applied Biotechnology, Faculty of Science and Technology, University of Westminster, UK
T.Keshavarz@westminster.ac.uk

Polyhydroxyalkanoates (PHAs) are biological polyesters produced through microbial fermentation processes. They have attracted attention as an alternative source to petro-chemically derived plastics as they are biodegradable, renewable, biocompatible and environmentally friendly. However, a notable limitation for their bulk production is the producer microbes' low yield and productivity which leads to high production costs. Intensive research is being carried out at all production steps including strain selection and improvement, media development, fermentation and bioreactor design to downstream unit operations in order to improve the overall process efficiency and performance. This review article concentrates on the current state of PHA production, with particular emphasis on media composition focusing on waste material as substrate. Bioreactor types and culturing methods will also be explored.

1. Introduction

Plastics possess a number of desirable properties over conventional substances. These include strength, durability, resistance to degradation and low density (Khanna and Srivastava, 2009). The problems associated with plastic accumulation in the environment and rapid-depletion of natural resources used in their production are motivating factors for research into sources and tools for alternatives to petroleum-based polymers.

Biopolymers are in general referred to as polymers produced by microorganisms under controlled conditions. They have many desirable functionalities and properties, including biocompatibility. Thus they find applications in the medical field. Many of the biomass-based biopolymers are biodegradable in addition to some petroleum-based biodegradable polymers which are also classified as biopolymers (Matsuura et al., 2008). Some are already industrially produced at a large scale (e.g. polylactic acid); however, many others are still to be optimized for commercial-scale production. A short classification of biopolymers is presented in Table 1.

Polyhydroxyalkanoates (PHAs) have attracted much attention in recent years due to their varied properties (thermoplastic and elastomeric), biocompatibility and biodegradability (Keshavarz and Roy, 2010). Bacteria synthesise PHAs as a carbon and energy source under the conditions of limiting nutrient(s) in the presence of an excess carbon source. Once the limiting nutrient environment is provided to the cells, these energy storage compounds are degraded and consumed. PHA exists as discrete inclusions that are typically 0.2 ± 0.5mm in diameter localized in the cell cytoplasm and can be visualised with a phase contrast light microscope due to their high refractivity (Khanna and Srivastava 2009).

Depending on the number of carbon atoms in the polymer chain, PHAs can be divided into two groups: short-chain length (scl) consisting of 3–5 carbon atoms and, medium-chain length (mcl), consisting of 6–14 carbon atoms. They are also classified as homo-polymer or hetero-polymer depending on whether one kind or more than one kind of hydroxyalkanoate is found as the monomeric units. Molecular weight of these polymers range between 2×10^5 and 3×10^6 Da depending on the micro-organism and the growth conditions (Keshavarz and Roy, 2010). Several bacteria have been reported to be capable of producing both scl- and mcl-PHAs. Scl-PHAs are stiff and brittle, whereas mcl-PHAs are more flexible. The ratio of the two types depends on the medium composition, mainly the carbon source.

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Table 1: Examples and classification of Biopolymers (Khanna and Srivastava, 2009).

Type of Polymer	***Name of Biopolymer***
Polyesters	Polyhydroxyalkanoates (PHAs)
	Polylactic acid
	Shellac
Proteins	Silk
	Collagen
	Elastin
	Resilin
	Poly-γ-glutamic acid
	Adhesives
Polysaccharides (bacterial)	Xanthan
	Dextran
	Gellan
	Polygalactosamine
	Cellulose (bacterial)
	Natural Rubber
	Levan
Polyphenols	Lignin
	Tanin
	Humic acid

Glucose has been found to be the most efficient substrate for the production of the scl-PHAs; however other substrates such as sucrose, methanol and acetic acid have also been used to produce scl-PHAs. Some organisms can grow on petroleum derived carbon sources such as alkenes, alkanes and aldehydes which actually act as precursor substrates for the production of structurally related mcl-PHAs (Francis 2011).

In terms of applications PHAs are used or have the potential to be used in various sectors. The possible application areas of PHAs are listed in Table 2 (Keshavarz and Roy, 2010).

In spite of the intensive research carried out on bacterial PHAs, their production cost is still far above the price of conventional plastics mainly due to the high cost of raw materials and relatively low conversion rates (Castilho et al., 2009). Several approaches are currently under investigation to make the process economically feasible and competitive. Some of these approaches include the development of recombinant microbial strains for a high substrate conversion rate (Nikel et al., 2006), more efficient fermentation process, better recovery and purification and the use of inexpensive substrates (Castilho et al., 2009). In this review the aim is to cover recent advances in the waste raw material, reactor types and culturing techniques recently used to enhance the production of PHAs and make them more cost efficient.

Table 2: Potential application areas of PHAs (Keshavarz and Roy 2010)

Area	***Use***
Packaging	Blow moulded bottles
	Milk cartons
	Cover for Cardboard
Food industry	Films
	flavour delivery agents
	food supplements
	diary cream substitutes
Medical	pericardial and atrial septal repair patches
	scaffolds
	vascular grafts
	cardiovascular stents
	heart valves
	drug delivery
Consumables	Moisture barriers
	Combs
	bullets
	Pens
	Sanitary towels
	Nappies
Chemicals	Bulk chemicals
	Compostable solvents
	Pressure sensitive adhesives

2. Raw Materials for Production of PHAs

Cost of raw materials, mainly carbon sources, is one of the most important factors affecting the overall economics of PHAs production specifically for large-scale process (Castilho et al., 2009). Therefore, the economic feasibility of bulk PHA production is intrinsically coupled with developing efficient fermentation processes from inexpensive carbon sources. Utilization of waste products as carbon sources present the advantage of concomitant decrease in disposal costs and production of value-added products (Du et al., 2012).

Increasing number of publications have discussed PHA production from different raw materials. Waste materials used for PHA production may be grouped into six categories: sugar-based media, starch-based media, cellulosic and hemi-cellulosic media, whey-based media and oil and glycerol-based media. The most common, inexpensive carbon source as an industrial waste material is molasses, either from sugarcane or beet.

Materials used for PHA production can be classified into six categories of sugar-based media, starch-based media, cellulosic and hemi-cellulosic media (Table 3), whey-based media and oil- and glycerol-based media (Table 4). The most common, inexpensive carbon source used as an industrial waste material is molasses, either from sugarcane or beet. Various strains have been evaluated for their capability to produce PHAs from beet molasses, sugar cane and date syrup. The highest PHA production reported is 23 g/L from *Azotobactervinelandii* (Page *et al*., 1992) followed by 22g/L by *Pseudomonas fluorescens* (Jiang *et al*., 2008) using molasses. Regarding other strains; it has been suggested that within the current technology it is less feasible to produce PHAs from *Bacillus sp*. because of their low production levels (Omar et al., 2001, Khiyami et al., 2011, Yilmaz and Beyatli, 2005).

Table 3: Sugar, starch, cellulose and Hemicellulose-based raw materials used on production of PHAs

Type	*Carbon Source*	*Organism*	*PHA Concentration (g/L)*	*Reference*
Sugar Based	Beet molasses	*Bacillus cereus*	0.16	(Yilmaz and Beyatli, 2005)
		Azotobacter vinelandii	23.00	(Page et al., 1992)
		Recombinant E.coli	9.00	(Liu et al., 1998)
	Sugar cane	*Pseudomonas fluorescens*	22.00	(Jiang et al., 2008)
	Date syrup	*Bacillus SA*	5.80	(Khiyami et al., 2011)
		Bacillus megaterium	1.50	(Omar et al., 2001)
Starch based	Soluble starch	*Bacillus cereus*	0.48	(Halami, 2008)
		Azotobacter chroococcum	25.00	(Kim, 2000)
	Potato starch	*Ralstonia eutropha*	94.00	(Haas et al., 2008)
Cellulose and hemi-cellulose based	Bagasse	*Burkhalderia sacchari*	2.73	(Silva et al., 2004)
		Burkhalderia cerpacia	2.33	(Silva et al., 2004)
		C.necator	3.90	(Yu and Stahl, 2008)
	Wheat	*C.necator*	51.10	(Koutinas et al., 2004)
	Wheat Bran	*Halomonas boliviensis*	4.00	(Van-Thuoc et al., 2007)
	Soy bean	*Recombinant E.coli*	4.40	(Lee et al., 1997)

Table 4: Whey, oil and glycerol based raw materials used on production of PHAs

Type	*Carbon Source*	*Organism*	*PHA Concentration (g/L)*	*Reference*
Whey Based	Whey	*Recombinant E.coli*	5.20	(Lee et al., 1997)
		Recombinant E.coli	32.00	(Kim, 2000)
		Recombinant E.coli	51.00	(Nikel et al., 2006)
		Recombinant E.coli	96.20	(Ahn et al., 2000)
		Recombinant E.coli	35.50	(Jae Park et al., 2002)
		Pseudomonas hydrogenovora	1.27	(Koller et al., 2008)
		Methylobacterium sp	6.12	(Koller et al., 2011)
		Osmophilic wild type strain*	5.50	(Koller et al., 2005)
Oil and glycerol based	Olive oil mill waste	*Recombinant P.putida*	0.13	(Ribera et al., 2001)
	Waste Glycerol	*C.necator*	38.10	(Cavalheiro et al., 2009)
		Osmophilic wild type strain*	16.20	(Koller et al., 2005)
	Soybean oil	*C.necator*	85-95	(Kahar et al., 2004)
	Waste frying oil (from rapeseed)	*C.necator*	1.20	(Verlinden et al., 2011)
	Canola oil	*W. Eutropha*	18.27	(López-Cuellar et al., 2011)

*Under characterization

Starch-based waste media are easily utilized by various microorganisms for PHA production. Haas *et al.* (2008) reported a PHA yield of 94 g/L using potato starch as substrate and *Ralstonia eutropha* as the producing microbe. Cellulose and hemi-cellulose-based waste materials have also been extensively studied. In 2011, Brazil as a top sugar producer, processed 625 million tons of sugarcane. Approximately 280 kg of humid bagasse is generated from 1 ton of sugarcane, as a waste material of sugar industry (Chandel *et al*., 2012). Silva *et al*. (2004) obtained 2.73 g/L PHA using bagasse as a cheap waste material; showing its potential for industrial biopolymer production. However within this group the most promising PHA production level of 51.1 g/L was obtained by Koutinas *et al*. (2004) using wheat as a raw material. Cavalheiro *et al*. (2009) and *Koller et al*. (2005) successfully utilized glycerol, a by-product of biodiesel production, for PHA production using *Cupriavidus necator*. They obtained a yield of 38 g/L and 16 g/L respectively. Different concentrations of PHAs obtained using oil-based waste materials are reported also (Castilho et al., 2009) with the highest production levels being 85-95 g/L using *Cupriavidus necator* (Kahar *et al*., 2004). Whey, among waste raw materials, is the most promising one due to its high nitrogen content and availability, making it an attractive substrate. It is the main waste material of cheese and casein production. Within the European Union approximately 40 million tons of cheese whey is produced annually and around 13 million of this remains unutilized (Koller et al., 2005). Cheese whey is mostly used in recombinant *Escherichia coli* cultures leading to the highest PHA production level of 96.2 g/L (Ahn et al., 2000).

3. Operation methods

The choice of operation strategy for production of bacterial PHAs depends on various factors including carbon source (defined e.g. glucose or complex waste material), culture (pure or mixed), mode of fermentation (batch, fed-batch, continuous), bioreactor type (air-lift reactor, and continuous stirred tank reactor (CSTR)).The fermentation may be carried out in a single stage or multi stages of sequencing batch system (SBR). Table 5 summarizes several processes employed recently with regards to the above mentioned factors; comparing them in terms of bacterial cell concentration, PHA concentration and productivity.

Batch fermentation for PHA production is a popular process due to its flexibility and low operation costs. However, it is associated with low PHA productivity since after utilization of the carbon source, bacterial cells degrade the accumulated PHA resulting in reduced PHA content (Zinn *et al*., 2001). Kulpreecha *et al*. (2009) reported a higher PHA productivity of 1.27 g/L/h compared to 0.45 g/L/h by *Bacillus megaterium* using sugarcane molasses under fed-batch compared to batch mode of fermentation. However, even though fed-batch fermentation, on its own, yields higher PHA productivity, the overall PHA production is still considered low in cases where nitrogen is the limited nutrient (Zinn et al., 2001). Batch and fed-batch processes are thus combined as a result of low PHA content obtained by each process individually. The combined process is the most common fermentation strategy used for PHA production. Under this strategy, the process is divided into two stages: in the first stage the microorganism is grown under batch mode until the desired biomass is achieved and PHA accumulation has started. In the second stage the fermentation is shifted to fed-batch, where usually one or more essential nutrients (most common is nitrogen) are maintained in limited concentration and carbon source is continuously fed into the reactor to further produce and accumulate PHA in the cells (Zinn *et al*., 2001). Verlinden *et al*. (2007) summarized several studies that employ a combination of batch and fed-batch systems in a two-stage process to encourage biomass production initially, followed by PHA accumulation and production. Likewise, Ibrahim and Steinbüchel (2009) reported a fed-batch fermentation of *Zebella denitrificans* at a pilot scale (42 L) using a stirred tank reactor (STR) for PHA production under improved aeration conditions.

In general pH and the % Dissolved Oxygen Tension (% DOT) in the reactor are maintained around 7 and 20% respectively; however the levels are adjusted based on the culture and the specific product. An example is the high cell density cultivation of *Pseudomonas oleovorans,* using n-octane as a carbon source in fed-batch culture. In this study Preusting et al. (2004) reported simultaneous cell growth and PHA accumulation by keeping pH at 7 and maintaining % DOT level between 30-40% by lowering the temperature of the culture broth to 18°C and the addition of nitrogen source at limited levels to the bioreactor. Consequently a high volumetric transfer rate was obtained (0.49 s^{-1}) accompanied by a final cell and PHA concentration of 37.1 and 12.1 g/L respectively.

Continuous culture, chemostat, is another option adopted as the third operation strategy for PHA production. In this method the culture broth is continuously replaced by sterile medium. In Chemostat culture, the carbon source is continuously fed in excess, keeping one or more nutrients (e.g. phosphorous or nitrogen) in limitation. Chemostat is highly controllable as the specific growth-rate can be maintained by adjusting the dilution-rate. Therefore under appropriate growth conditions, continuous fermentation might

have the potential to give highest PHA productivity levels. Nonetheless chemostat culture exhibits a higher risk of contamination (Zinn et al., 2001).

In terms of bacterial cultures used, majority of the reports emphasize the use of a single bacterial strain employing different operation systems and reactor types, and a variety of cheap substrates (sugarcane molasses, waste potato starch, hydrolysed corn oil) or other organic compounds such as glycerol or glucose for PHA production. Studies of this type were mainly carried out at bench-scale utilising a CSTR to either maintain the culture at log phase by constant introduction of the feeding medium and removal of the culture solution (continuous fermentation) or batch fermentation of an organic matter under anaerobic condition followed by utilization of an air-lift reactor for PHA production. The choice of strain also appears to influence the operation mode used for PHA production; Ishizaki *et al*. (2001) reported a significantly higher PHA production in a fed-batch culture of *Ralstonia eutropha* compared to its cultivation in continuous mode. Considering this, they suggested the use of *Alcaligenes eutrophus* instead when continues mode was employed. This was based on the finding that the latter organism was able to accumulate P(3HB) during the exponential phase.

Salehizadeh and Van Loosdrecht (2004) reported in their review the use of mixed cultures for biopolymer production with reduced overall process cost. The process involves few steps of enriching the culture and utilization of substrate followed by batch fermentation for PHA production. They suggested a Sequencing batch reactor (SBR) for the industrial production of PHA in batch/fed batch mode, or a plug flow reactor (PFR) followed by a continuous stirred tank reactor (CSTR) if a continuous system is to be employed. Other reports were also published subsequently emphasising the use of mixed cultures in order to enhance the PHA productivity. Figures of up to 75% are reported, employing a three step process. At bench scale the SBR commenced with anaerobic fermentation followed by enrichment of the culture using a fed batch system in a CSTR and then a batch PHA accumulation step in a STR (Albuquerque et al., 2007). Other reports are also available on using other fermenter types such as bubble-column reactor for PHA accumulation using both batch and fed-batch processes (Preusting et al., 2004).

Table 5: *Comparison of current processes used for PHAs production*

Organism	Carbon source	Bioreactor (V/WV)	CT (h)	Stages	Fermentation Process Type	Cell Conc (g/L)	Prod (g/L/h)	References
P. aeruginosa	Sugarcane molasses	7.5/2.5L STR	54	Single	Batch	7.32	0.11	(Tripathi et al., 2012)
R. eutropha	Dual: Stage 1:CCS Stage 2: Fed with ARF	5/4L STR	144	Two	Batch/Fed-Batch	21.13	0.0697	(Chakraborty et al., 2012)
C. necator	Glycerol	3/2L STR	60	Single	Fed-Batch	75	0.92	(Tanadchangsaeng and Yu, 2012)
P. putida	Hydrolysed corn oil	30 L Jar fermenter	46	Single	Fed-Batch	103	0.61	(Shang et al., 2008)
C. necator	Glucose	5 CSTR Growth phase: Reactor 1: 7.5-L Production phase: Reactors 2-5: 3.6L	34	Multiple	Growth phase : Batch Production phase: continuous (D= 0.139 /h)	81	1.85	(Atlić et al., 2011)
P. putida	MM continuously fed with SO	OBB (WV=1.5L)	48	Single	Continuous	3.75	n/a	(Troeger and Harvey, 2009)
P. putida	Oleic acid	5/3.3L STR	70	Single	Fed-Batch	30.22	0.1878	(Marsudi et al., 2009)
Bacterial consortium	Sugarcane molasses	AF: anaerobic CSTR (1.14L) Culture selection :SBR (WV:1L) PHA accumulation: Batch (0.6L)	n/a	Multiple	Stage 1: Continuous Stage 2: Feast & Famine Stage 3: Batch	2-3	0.43	(Albuquerque et al., 2007)
B. megaterium	Sugarcane molasses Fed with MSM, Cane molasses and urea	5/2.5 L Jar fermenter	24	Single	Fed-Batch	72.6	1.27	(Kulpreecha et al., 2009)
B. megaterium	Sugarcane molasses	5/3 L Jar fermenter	12	Single	Batch	8.78	0.45	(Kulpreecha et al., 2009)
W. eutropha	Fructose	Glass bioreactors (1.5/4 L)	n/a	Two	Continuous (D= 0.1/ h)	3.75	n/a	(Khanna and Srivastava, 2007)
P. putida	Glucose- Fed with nonanoic acid & 10-undecenoic acid	5L STR	25	Single	Exponential Fed-batch	33.6-54.1	0.63–1.09	(Sun et al., 2009)
P. putida	Oleic acid	5L:3.3L STR	70	Single	Fed-Batch	30.22	0.1878	(Marsudi et al., 2009)
R. eutropha	Waste potato starch	5/3.4 L	70	Single	Fed-Batch	179	1.47	(Haas et al., 2008)
A. eutrophus	Glucose	n/a	50	Single	Fed-Batch	164	2.42	(Kim et al., 1994)

Prod=productivity, (v/wv) volume/working volume,CCS= Condensed corn solubles,ARF= Artificial rumen fluid,CT= cultivation time (h),SBR= sequencing batch reactor, MSM= mineral salt medium, MM= mineral medium, D= dilution rate, OBB= Oscillatory Baffled Bioreactor, STR=stirred tank reactor.
AF: Acidogenic fermentation, SO: sodium octanoat

4. Conclusions

Considering current advances in biopolymer research, PHAs have shown great potential as a replacement for petroleum-based plastics. The challenge for the future application of the PHA polymers depends mostly on the increase in the production levels of these polymers with the desired various properties in an economical fashion. There is room for improvement of the current technology for the whole process from the start to the final step. This suggests the selection and development of bacterial strains that are capable of efficient consumption and transformation of various substrates into a range of PHAs with different properties, at high yield and productivity; high performance fermentations, and efficient extraction and purification to lower the price. While engineering recombinant bacterial strains that utilise cheap carbon substrates with high conversion rate need to be taken into consideration; their stability is an important factor for successful PHA production. Up to date, among the investigated waste material, whey seems to be the most promising using recombinant microorganism. Regarding the cultivation processes, combining batch and fed-batch fermentations has given the highest productivity compared to the other reported methods. However, considering the controllable nature of chemostat, it has the greatest potential to provide higher productivities. This field of research requires further investigation in future to enhance the productivity and lower the production costs to make it more competitive. All efforts at laboratory scale will need to be validated at pilot-scale for future industrial production. The challenges of scale-up process might put a question mark against those procedures and processes that have been proposed to be promising.

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