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Efficient biosynthesis and recovery of polyhydroxyalkanoate

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ABSTRACT

Petrochemical-derived plastics have become a source of pollution for decades, and finding alternative plastics that are environmentally friendly has become a matter of urgency. Polyhydroxyalkanoate (PHA), a biopolyester synthesized by microbial cells, has properties that make it suitable as a biodegradable plastic material. The diversity of PHA makes it applicable to a wide range of products, from packaging to biomedical devices. The main challenge in commercialization of PHA is the cost of production. Although many studies have been focused on obtaining high yields of PHA, up until now, there is no absolute definition of efficient production of PHA, as there are many factors that could contribute to the efficiency of a process. Efficiency in PHA recovery also contributes to the commercial viability of PHA production. This review focuses on the efficiency of PHA biosynthesis from several aspects relating to the criteria for efficient production. The development of new strategies for improved production, including utilization of low cost carbon sources, genetic modification of PHA-producing microbes, and fermentation strategies are discussed here. Advances in recovery of PHA, as well as the potential of biological recovery techniques, are also highlighted in this review.

Keywords: Efficiency, biosynthesis, polyhydroxyalkanoate, recovery

INTRODUCTION

Since the advent of mass manufacturing, synthetic plastics have become essential to human lives while simultaneously becoming the bane of the environment. Despite efforts to manage plastic waste, large quantities of plastic materials are disposed uncontrollably in terrestrial and aquatic environments. Plastics are non-biodegradable, but can be fragmented into microplastics, i.e. less than 1 mm in size (Van Cauwenberghe *et al.*, 2013). Organisms, especially marine animals, can easily ingest these microplastics due to their small size and cause bioaccumulation in any food web. Plastics or their derivatives may eventually end up in the human body by consumption of other organisms grown in plastic-polluted environments (Hammer *et al.*, 2012). As it is almost impossible to remove all traces of plastics upon disposal, the collective resolution is to reduce or eliminate the source of plastic waste. This was further emphasized by the recent ban on microbeads in the United States (Rochman *et al.*, 2015).

Regardless of the alarming state of plastic pollution, the demand for plastics continues to rise along with the increase in global population. As shown in Figure 1, global plastics production in 2013 was 299 million tonnes, which was an increase of more than 40 million tonnes from 2009 (PlasticsEurope, 2015; World Bank, 2016). In addition to the challenges in waste management, plastic production also contributes to the depletion of non-renewable resources. Approximately 4% of fossil fuel obtained worldwide is channeled into feedstock for plastic production. Energy consumption during the manufacturing process accounts for an additional 3 to 4% usage of oil and gas (Hopewell *et al.*, 2009). Thus, there is an urgent need to look into alternative plastic materials, which can be produced in a sustainable manner.

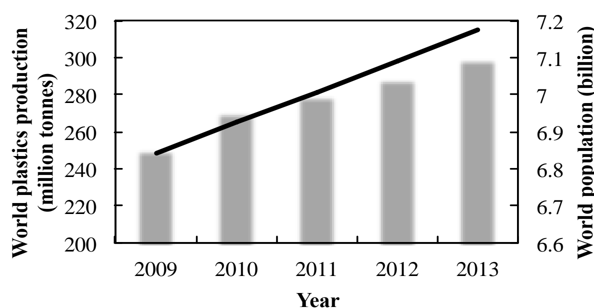

Figure 1: Global plastics production from 2007 to 2013. (Adapted from PlasticsEurope, 2015; World Bank, 2016).

Research on biopolymers has become more intensive over the years. Biopolymers can be broadly categorized as polymers obtained directly from biological materials, such as proteins and polysaccharides; polymers derived from bio-based monomers, such as polylactic acid (PLA); and polymers synthesized biologically by wild-type or

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genetically-modified microorganisms, such as bacterial cellulose and polyhydroxyalkanoates (PHA) (Robertson, 2013). PHAs possess polymeric properties comparable to those of polyethylene (PE) or polypropylene (PP) and have generated great interest as a suitable alternative to these conventional plastics (Visakh, 2014). In 2014, 35 thousand tonnes of PHA was produced, signifying a 9% compound annual growth rate (CAGR) from 2013. Furthermore, there were 16 PHA-manufacturers in 19 locations with expected production up until 2020 (Aeschelmann and Carus, 2015).

Polyhydroxyalkanoate is a type of polyester produced intracellularly by microorganisms as a storage compound when grown in environments with nutritional limitations, such as nitrogen, but when carbon sources are present in excess (Anderson and Dawes, 1990). This response has been investigated in a wide range of bacterial strains and the PHA biosynthesis pathway has been well characterized. With the long-term goal of replacing petrochemical plastics with PHA, this PHA-accumulating ability in microorganisms has been exploited to produce high yields of PHA from various renewable carbon sources or to produce specific types of PHA. Economic feasibility of industrial-scale PHA production ultimately depends on the efficiency of the process from start to finish, including the recovery of the polymer. In this review, we will discuss the concept of efficiency in relation to PHA production, taking into consideration the factors that contribute to efficient production of other industrially significant biopolymers. We will also review the methods and strategies employed in notable studies on efficient PHA production. In addition, the downstream processes involved in PHA production and the prospect of biological recovery to enhance efficiency will be highlighted.

CONCEPT OF EFFICIENCY

What does efficiency mean?

White Biotechnology or also known as Industrial Biotechnology involves the use of living cells or enzymes to create industrial products with enhanced performance, that are easily degradable, require less energy and create lesser waste during production as compared to products produced using traditional chemical processes. In this case, the efficiency or the practicality of the processing method that leads to the final output is essential for the sustainability of industries. The term efficiency denotes a measure or ability to circumvent the waste of materials, energy, efforts, and time in doing something or in producing a desired result. In other words, it is the ability to produce a product successfully and without waste, which involves the capability of a specific application or process to generate the specific outcome with a minimal amount of cost and energy.

A process is deemed efficient if the following factors (Figure 2) could be achieved:

i. Time: Less time is needed to generate yield of a certain product. Processing steps are reduced.
ii. Productivity: Increase in production with effective processing method.
iii. Cost: Expenses on labour, energy and materials are cut down. The use of biomass feedstock and waste as substrate to replace costly and synthetic substrates. Transforming low value waste into high value product.
iv. Recovery: The product could be recovered without the excess use of chemicals and the processing method is more environmentally friendly.
v. Stability: The product generated is stable and does not disintegrate easily. Its characteristics are stable throughout the processing time.
vi. Commercial viability: The product has the desired properties and quality, with high market demand, profitable and safe to be used.
vii. Sustainable: Supply of feedstock, usage of energy and market demand for the product are sustainable over a reasonable time period.

A complete conversion of the input to output with the desired properties and commercial viability is the most ideal framework of efficiency. Therefore, continuous efforts are needed to achieve this goal because efficiency changes with time. What was considered efficient 10 years ago may no longer be competitive now. Research has been ongoing to increase the efficiency of production of beer, wine, and alcohol, biodiesel, washing detergent, sugar, plastics, fabrics, personal care products, biogas, bioethanol, food and drink as well as healthcare products.

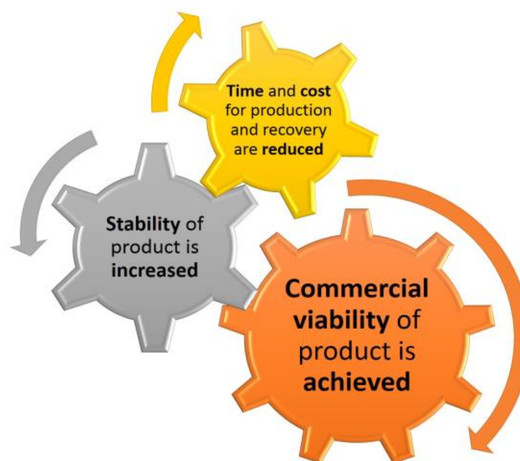

Figure 2: The factors that contribute to the efficiency framework. The importance of the factors is defined by the size of the gear.

Biotechnological products with industrial importance

Here, a few examples of notable leading companies that have achieved high efficiency in their productions and biotechnological products with industrial importance are described. To date, DSM (Het Overloon 1, 6411 TE Heerlen, Netherlands) is one of the global leaders in white biotechnology with a fermentation network comprising of

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13 plants worldwide and a total fermentation capacity of over 30 million cubic meters per year. DSM collaborated with a French company Roquette (62080 Lestrem cedex, France) to produce bio-succinic acid, which shows a better performance than chemically produced succinic acid with the lowest eco-footprint (up to 50% reduction of greenhouse gas emission) and zero solid waste production. White biotech-based advanced fermentation technology at the antibiotics plants in Delft, Netherlands has successfully replaced a complex 13-step chemical process with a one-step fermentation, two-step enzyme process, while saving energy up to 65% and reducing the costs of raw material to half. Meanwhile, DSM together with DONG Energy (Denmark) have demonstrated the combined fermentation of C6 and C5 sugars from wheat straw on an industrial scale. They found that the combined fermentation results in a 40% increase in ethanol yield per ton of straw, which significantly reduces the cost of production of bioethanol from cellulosic feedstock (DSM, 2011).

Pfizer (235 East 42nd Street NY, NY 10017) is a leading company that has successfully produced large amounts of penicillin by utilizing deep-tank fermentation, surpassing the cultivation difficulty and instability of the small quantities produced. Besides that, Pfizer also managed to produce itaconic acid, fumaric acid and other intermediates for chemical synthesis through fermentation processes. Meanwhile, gluconic acid was produced efficiently after a commercial process involving deep-tank fermentation was established. The significant breakthrough for Pfizer lies in the production of gluconic acid and other products using deep-tank fermentation. The successful mass production of penicillin was inspired by the development of deep tanks for gluconic acid (ACS National Historic Chemical Landmarks, 2008).

Another classical example is the production of baker's yeast. Baker's yeast which is from the *Saccharomyces cerevisiae* species has been widely used as the leavening agent in bread making and bakery products. The importance of baker's yeasts in the baking industry has drawn the efforts to develop and market yeasts with reliable performance in large scale. It was reported that the bread industry and the yeast industry were growing together since they are interdependent (Frey, 1930). Apart from baker's yeast, brewer's yeast is used in the fermentation of alcohols such as beer and winemaking.

Biodiesel has become an attractive alternative to diesel fuel as it is made from renewable vegetable oils and animal fats. It is also environmentally favorable due to its properties such as biodegradable, nontoxic and low emission profiles (Krawczyk, 1996). Due to the increasing demand, microalgae such as *Chlorella* sp. has been used to produce biodiesel. Although microalgal biodiesel does not adhere strictly to the criteria of being industrially viable, nor has it been established as a commercial product, oil productivity of many microalgae was proven to exceed the oil productivity of the best producing crops significantly. Furthermore, microalgae has higher photosynthetic efficiency, higher biomass production and faster growth as compared to other energy crops (Milne *et al*., 1990; Ginzburg, 1993; Dote *et al*., 1994; Minowa *et al*., 1995). Miao and Wu (2006) reported that biodiesel comparable to conventional diesel was obtained from heterotrophic microalgal oil by acidic transesterification. In their study, the heterotrophic growth of *Chlorella protothecoides* resulted in 55% of accumulation of high lipid content in the cells and large amount of microalgal oil was efficiently extracted from these heterotrophic cells. Many approaches for making biodiesel more commercially viable have been taken such as through genetic and metabolic engineering, and the use of biorefinery concept. It was shown that photobioreactors could provide a controlled environment that can be tailored to the specific demands of highly productive microalgae to attain a good annual yield of oil consistently (Chisti, 2007). Nevertheless, low cost microalgal biodiesel is yet to be achieved.

Emergence of polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) have emerged as a biotechnological product that is capable of becoming a valuable commodity. Synthetic plastic wastes have long been known to cause harmful effects to the environment due to its non-degradability. On the other hand, PHAs are biological polyesters that are biodegradable, renewable, biocompatible, and environmentally friendly. PHAs have properties similar to numerous elastomers and thermoplastics; it can also degrade completely in the environments (Holmes, 1988; Steinbüchel, 1991; Steinbüchel, 1992; Lee, 1996a). Thus, it could serve as an alternative to petrochemical-derived plastics. Efforts have been carried out in order to reduce the PHA production cost, such as by developing better bacterial strains and more efficient fermentation/recovery processes (Lee 1996a; Lee, 1996b).

Polyhydroxyalkanaote productivity is calculated based on the amount of PHA produced by unit volume in unit time (g/L·h). For low productivity processes, larger equipment is needed in order to produce the same amount of PHA per year. In a hypothetical scenario, a fermentation process could produce 100 g PHA/L in 50 h (a productivity of 2 g PHA/L·h). To produce 10 tonnes of PHA in 250 h (without taking into consideration the recovery step), a fermentor with a working volume of 20 m^3 is required. On the other hand, if in 50 h, only 50 g/L PHA could be achieved (productivity of 1 g PHA/L·h), a larger fermentor (double in size) is required in order to obtain the similar output (Choi and Lee, 1999). Several factors could affect the economics of PHA production such as the cell biomass productivity, the yield of PHA and the PHA content, the cost of raw materials and carbon sources used as well as the efficiency of the recovery method.

Apart from that, PHA content could also affect the efficiency of the recovery process and the PHA yield. After all, the purity and recovery yield of PHA are interrelated and highly dependent on the PHA content. It was reported that a relatively low P(3HB) content of 50 wt% of the cell dry weight (CDW) led to a costly recovery

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($ 4.8/kg P(3HB)) as compared to the recovery cost process of 88 wt% P(3HB) content [$ 0.92/kg P(3HB)] (Lee and Choi, 1998). Likewise, the cost of carbon source will also determine the overall production cost of PHA (Yamane, 1992; Yamane, 1993). The use of cheaper carbon sources as feedstock could lower the production cost of P(3HB). Experimental high level yields of PHA production from glucose and vegetable oils (fatty acids) was reported to be in the range of 0.3 to 0.4 g P(3HB)/g of glucose (Ryu *et al*., 1997) and 0.6 to 0.8 g PHA/g of oil (Naylor and Wood, 1999), respectively. By using *Cupriavidus necator*, high yields of P(3HB-*co*-3HV) ranging from 0.64 to 0.72 g PHA/g of oil was produced from rape seed oil or corn oil together with propionic acid. Kahar and colleagues have also reported that the yield of PHA from soybean oil was in the range of 0.72 to 0.76 g/g and the PHA productivity was approximately 1 g/L·h (Kahar *et al*., 2004). Akiyama and coworkers have proposed a stoichiometric equation whereby about 1.38 g of P(3HB) could be produced from 1 g of linoleic acid (main fatty acid composition in soybean oil) (Akiyama *et al*., 2003).

Besides that, efficiency could also be evaluated from the aspect of environmental friendliness. Although PHAs are produced from renewable resources and could degrade naturally, a discourse exists regarding the actual environmental impact of this material. Thus, it is essential to assess the life cycle of PHA. In 2003, Akiyama and colleagues have taken the initiative to compare the environmental life cycle of PHAs produced from renewable carbon sources by bacterial fermentation. The study attempted to predict the fermentation production cost of a PHA copolyester using soybean oil as carbon source, with the assumption that the recombinant strain of PHA-negative *C. necator* harboring the *A. caviae* PHA synthase gene would produce P(3HB-*co*-5 mol% 3HHx) on a large commercial scale, i.e. up to 5000 tonnes per year. In addition, the cost and life cycle inventories for the fermentation production of P(3HB) from glucose were also estimated and used for assessment. They estimated that the annual production of 5000 tonnes of P(3HB-*co*-5 mol% 3HHx) will cost from 3.5 to 4.5 US$/kg, depending on the presumed production performances. Similar scale production of P(3HB) from glucose was estimated to cost 3.8 to 4.2 US$/kg. Life cycle inventories of energy consumption and carbon dioxide emissions favor the production of P(3HB-*co*-5 mol% 3HHx) over P(3HB), reflecting smaller inventories and higher production yields of soybean oil as compared to glucose. It was discovered that the life cycle inventories of energy consumption and carbon dioxide emissions of bio-based polymers are significantly lower than the petrochemical polymers (Akiyama *et al*., 2003).

Experimental and industrial manufacturing of all types of PHAs were initiated by small and middle-sized companies. Some of the larger companies such as Meredian (U.S), Ecomann (China) and Bio-on (Italy) have put much effort in increasing the production capacities. Nevertheless, high prices and performance issues remain the two major constraints for PHA market. Despite the challenges faced by the PHA industry, Biopol®, Nodax™ and DegraPol® managed to be commercialized and survive in the market. Biopol®, a thermoplastic copolymer of P(3HB-*co*-3HV) has a melting point of 140 to 180 °C. Nodax™ is made up of 3-hydroxybutyrate and a relatively small quantity of MCL monomers with the side groups of three carbon units or more, while DegraPol is a block-copolyester urethane chemically synthesized from P(3HB)-diol and α, ω-dihydroxy-poly(ε-caprolactone-block-diethyleneglycol-block-ε-caprolactone).

Biopol® was found to be suitable for injection, blow moulding and film production. It has been used for paper and paperboards coating, for electric and electronic packaging. Due to its favourable properties, companies such as Fluka and Toray have shown emerging interest in utilizing Biopol® for medical applications (Clarinval and Halleux, 2005). In 1992, Brocato International (Baton Rouge, LA, USA) marketed their hair care products using Biopol® bottles. Wella, the German haircare company, also packaged their shampoos, Sanara Extra Mild Care and Linie N Marigold using Biopol® bottles. Apart from that, Biopol® filaments have also been used in the manufacturing of fishing nets and ropes while its fibers were used to make ropes and nets for crab cages due to its excellent durability and biodegradability in the sea. It was reported that Biopol® with polyvinyl alcohol coating is used for growing seaweed due to its good matrix. Furthermore, Biopol® fortified with polycaprolactone (PCL) was shown to develop anti-algal properties which can be used in nets for seafood cultivation (Asrar and Gruys, 2002). Biopol® is therefore one of the most tested PHA in various products and applications.

On the other hand, Nodax™ is another PHA copolymer that has been tested in the development of biodegradable foams, fibers, latex, films and nonwoven fabrics. This copolymer possesses both anaerobic and aerobic degradability along with hydrolytic stability, elasticity and mechanical properties. It could also be made into flushable hygienic wipes and tampons, which are able to degrade in the septic systems. Besides that, its potential applications have also been tested in the manufacturing of compostable bags and lids or tubs for thermoformed articles, surgical attires, upholstery, mats, and for packaging (Noda *et al*., 2005).

There are many benefits that PHA can offer. In order to ensure the sustainability of PHA in the long run, all the factors that determine the efficiency of PHA production and recovery need to be thoroughly evaluated and optimized.

TOWARDS THE PRODUCTION OF COMMERCIALLY COMPETENT PHA

Production of P(3HB) from low-cost substrates

As the most well-studied PHA, there has been rapid development of efficient processes to manufacture P(3HB). With regards to financial feasibility, the use of inexpensive carbon feedstock is an important aspect to consider. Simple sugars such as fructose and sucrose are

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easily obtained from plants; however, these sugars are used as a food source and would contradict the purpose of lowering PHA production costs if used as a feedstock. Cellulosic materials from plant biomass are the most abundant carbon source and do not compete with human consumption. With this potential in mind, intensive research is ongoing to develop more facile and cost-effective methods to obtain sugars from lignocellulose.

In a study by Cesário and colleagues, wheat straw was pretreated by ammonia fiber expansion method (AFEX) before enzymatically hydrolysing the cellulose and hemicellulose fractions (Cesário *et al.*, 2014). The resulting hydrolysate contained approximately 50 g/L sugars, which were predominantly glucose, followed by xylose and arabinose. The hydrolysate as-is was fed to *Burkholderia sacchari* (DSM 17165) and yielded CDW of 7.0 g/L in shake flasks, with 34 wt% P(3HB) content. Different sets of concentrated hydrolysate with sugar concentrations ranging from approximately 890 g/L to 1115 g/L were used for scaled-up fed-batch fermentation. The maximum CDW obtained was 140 g/L and the highest P(3HB) content was 68 wt%. The maximum P(3HB) productivity was 1.5 g/L·h after 39 h of cultivation, which is the highest productivity reported for fermentation on lignocellulosic hydrolysates. From their study, improvement in cell growth was observed by using the hydrolysate with lower citrate content. Although citrate may serve as an added nutritional component, the authors have found that the consumption of citrate interferes with pH control during the fermentation process.

The use of lignocellulosic hydrolysates in fermentation has several other challenges, including the presence of inhibitory compounds. The use of hydrolysates may require detoxification steps to remove inhibitors such as furfurals, 5-hydroxymethylfurfural, or phenolic compounds. Various methods of detoxification have been studied, leading to improved PHA production (Pan *et al.*, 2012). However, this would contribute to higher processing cost. Some researchers have explored the potential of certain bacterial strains to grow and produce PHA in the presence of typical lignin derivatives (Dietrich *et al.*, 2013; Tomizawa *et al.*, 2014; Numata and Morisaki, 2015). A recent study by Heng *et al.* (2016) highlighted the importance of selecting the right bacterial strain for efficient utilization of hydrolysates, due to their varying tolerance towards inhibitory compounds that are potentially present in biomass hydrolysates. In their study, *C. necator* NSDG-GG, a mutant strain of *C. necator* H16, grew better on synthetic medium than *Burkholderia cepacia* USM (JCM 15050), but did not grow as well on enzymatic hydrolysate of rice husks. However, the converse was observed for *B. cepacia* USM, whereby cell growth was markedly improved when fed with the hydrolysate.

Besides lignocellulosic biomass, waste materials from food manufacturing industries have also been investigated for their potential as an inexpensive carbon source. For example, corn steep liquor is a residue from the wet milling of corn that is rich in nutrients such as amino acids and minerals. It has long been recognized as an inexpensive medium for microbial growth since it was popularized as a growth medium for penicillin production (Liggett and Koffler, 1948). Another material is whey, which is rich in lactose and is an abundant waste from the dairy industry. In fact, disposal of whey has become a matter of concern in Europe; the dumping of whey on land and in water has been made illegal due to its acidity. Although it can be recycled as a feed for cows, it is not suitable as complete fodder as it causes digestive problems in the animal (Elliot, 2013). Therefore, the use of such materials as a carbon source for PHA would not only reduce the cost of production, but would also alleviate the problem of waste disposal faced by food manufacturers.

Sugarcane molasses is a by-product of the sugarcane industry that is still being sold as a food product, but are low in value due to its low purity. However, these impurities can serve as growth factors for microbial cultivation, similar to corn steep liquor. Using sugarcane molasses, Kulpreecha and colleagues were able to perform fed-batch fermentation for P(3HB) production by *Bacillus megaterium* BA-019 (Kulpreecha *et al.*, 2009). The molasses supplied contained up to 400 g/L of total sugar, and were fed by a pH-stat strategy to maintain the pH at 7.0. CDW of 72.6 g/L with 42 wt% P(3HB) content was obtained in a cultivation duration of 24 h, resulting in a volumetric productivity of 1.27 g P(3HB)/L·h. Comparatively, production of P(3HB) from another strain of *B. megaterium* from sugarcane molasses supplemented with corn steep liquor as the nitrogen source yielded CDW of only 1.46 g/L and P(3HB) productivity of 0.54 g/L·h after 12 h of batch fermentation (Gouda *et al.*, 2001).

An interesting waste material, spent ground coffee, contained up to 15 wt% coffee oil and was found to have a high content of fatty acids (Obruca *et al.*, 2014). The extracted coffee oil resulted in the highest CDW (14.2 g/L) and P(3HB) content (70 wt%) in *Cupriavidus necator* H16 when compared with other waste oils at shake-flask scale. Batch fermentation with 30 g/L of coffee oil resulted in CDW and PHA concentration of 29.4 g/L and 26.5 g/L respectively, with a final P(3HB) productivity of 0.66 g/L·h. Further improvements were observed in fed-batch fermentation with pulsed feeding of the coffee oil, yielding up to 55 g/L CDW and 49.4 g/L P(3HB) concentration. A total of 60 g/L coffee oil was fed throughout 37 h. The cells were able to accumulate P(3HB) up to 89 wt% in fed-batch fermentation, which was similar to 90 wt% in batch fermentation. However, the volumetric productivity increased approximately two-fold to 1.33 g/L·h. The authors have also suggested that the solid coffee residue that remained upon oil extraction could be further utilized as an energy source via combustion, thus maximizing the utility of this by-product.

Even with the current strategies employed to bring down the cost of feedstock, large-scale PHA production will reach another bottleneck if the organism of interest fails to utilize the feedstock efficiently. It has been suggested that genetic modification on microbial strains may be the simplest and most economic approach

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towards industrial production (Peralta-Yahya and Keasling, 2010). Advancements in molecular biology are able to accelerate the engineering of highly efficient PHA-producing strains at a lower cost. Together with molecular approaches, rapid developments in synthetic and systems biology also contribute significantly to the holistic understanding of metabolic systems in microbial cells, enabling researchers to dictate the metabolic functions of the cells for synthesis of desired products.

Wild-type *Escherichia coli* cannot synthesize PHA, but its ease of genetic engineering has enabled *E. coli* strains to be transformed with plasmids carrying genes specific to the PHA biosynthesis pathway. PHA pathway construction has led to recombinant *E. coli* strains capable of growing on a wide range of inexpensive substrates with high yields of PHA. Pioneer studies on recombinant *E. coli* expressing the biosynthesis pathway of P(3HB) from *C. necator* (formerly known as *Alcaligenes eutrophus*) found that the recombinant strains were able to accumulate high amounts of this homopolymer (Schubert *et al.*, 1988; Slater *et al.*, 1988). In a separate study, further manipulation was carried out on recombinant *E. coli* to enable expression of the P(3HB) synthase gene under new transcription and translation conditions, i.e. utilization of the T7 gene 10 ribosome-binding site to initiate translation. P(3HB) accumulation up to 70 wt% of the CDW was achieved when grown on a glucose-free Luria-Bertani (LB) medium (Kalousek and Lubitz, 1995).

Essentially, cloning and expression of the PHA operon is a facile method to convert non-PHA producers into PHA producers, but high yield and volumetric productivity depend on many other factors. A study by Lee and coworkers (Lee *et al.*, 1994) using recombinant *E. coli* harbouring *C. necator* PHA biosynthesis genes reported high P(3HB) productivity of 2.08 g/L·h by fed-batch fermentation in medium containing yeast extract and tryptone. However, these results could not be reproduced when a chemically defined medium was used and this problem was attributed to the filamentation of the cells. Filamentation is a form of pleomorphism observed in *E. coli* when grown in stressed conditions, whereby the cells prepare to undergo cell division but remain conjoined, resulting in an elongated morphology. It is also commonly observed in *E. coli* mutant strains (Wainwright *et al.*, 1999). To overcome filamentation, insertion of a plasmid harbouring both the PHA biosynthesis genes and *fts*Z gene from *E. coli* enabled the overexpression of *fts*Z, which suppressed filamentation and allowed the cells to reach a P(3HB) productivity of 2.8 g/L·h in 50-L scale fermentation using a defined medium (Lee, 1994; Wang and Lee, 1997).

In another study, the *phaBAC* genes from *Azotobacter* sp. strain FA8 were transformed into *E. coli* strain K24K and expressed under the control of the *lac* operator (Nikel *et al.*, 2006). When the *lac* repressor is present, it binds to the *lac* operator and prevents the transcription of genes under its control. The strain was unable to produce the *lac* repressor, thus ensuring the constant uptake and transport of lactose into the cells. By placing the PHA biosynthesis genes under the control of the *lac* operator, this recombinant strain was able to express the PHA genes without the addition of an inducer and was able to accumulate PHA from whey, a lactose-rich feedstock. Plasmid stability in the recombinant strain was improved with the addition of a kanamycin resistance gene, and this improvement was also reflected in the PHA content, which increased from 30 wt% to 73 wt% of the CDW. Fed-batch fermentation on a medium containing two waste materials, i.e. whey and corn steep liquor, for 24 h resulted in a volumetric productivity of 2.13 g/L·h of P(3HB).

Once a strain has been tailor-made for efficient PHA production via genetic modifications, the next challenge is to provide the appropriate conditions that would allow it to synthesize PHA optimally. Such an effort has been exemplified by Ahn and colleagues, whereby a recombinant *E. coli* GSC 4401 transformed with a pJC4 plasmid harbouring *A. latus* PHA biosynthesis genes was found to accumulate P(3HB) up to 96.2 g/L on concentrated whey, resulting in volumetric productivity of 2.57 g/L·h over a period of 37.5 h when a pH-stat fed-batch fermentation process was employed (Ahn *et al.*, 2000). In their study, they investigated the problems associated with using whey as a feedstock, particularly the poor solubility of lactose and volumetric restrictions of the fermentation vessel that required removal of the culture medium during fermentation. To concentrate the whey solution beyond the solubility of lactose in water (210 g/L), the whey was pretreated to remove excessive proteins and a final concentration of 280 g lactose/L whey solution was obtained. Moreover, the control of dissolved oxygen concentration (DOC) throughout the fermentation was found to be extremely crucial. Supplying DOC at sufficient levels during the stage of active cell growth was necessary to achieve high cell density; in the PHA synthesis stage, lowering the DOC in a step-wise manner triggered rapid accumulation of P(3HB).

Further enhancements were made to the fermentation process using a cell recycle strategy with the aid of an external membrane unit, which enabled the working volume to be maintained at a specific set point (Ahn *et al.*, 2001). Starting with a 1.3 L working volume in a 6.6-L jar fermentor, pH-stat feeding proceeded up to a volume of 2.3 L. The culture volume was maintained by removing an equal volume of feed, while the cells were circulated back into the fermenter using a cross-flow type external membrane made of polysulfone hollow fibers. The cell reycle strategy was initiated upon depletion of lactose, which was indicated by an increase in pH. Using concentrated whey as feedstock, volumetric productivity was enhanced by almost 2-fold, reaching 4.6 g/L/h in 36.5 h, which is the highest report of P(3HB) productivity thus far.

Limitations of P(3HB)

Although numerous studies reported in literature have successfully demonstrated the possibility of highly efficient P(3HB) production, the polymeric properties of

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this homopolymer have conferred some disadvantages to its application as a commercial plastic material. Specifically, P(3HB) is brittle due to its crystallinity. It also has a melting temperature that is close to its degradation temperature, thus limiting its processivity (Doi, 1990). Previously, the incorporation of the 3HV monomer was believed to enhance the flexibility of the polymer, as evidenced by its commercialization in the 80's. However, depending on the molar fraction of 3HV, the crystallinity of P(3HB-*co*-3HV) was not different from that of P(3HB), due to the co-crystallization of the monomers in the polymer lattice (Kamiya *et al*., 1991; Pan and Inoue, 2009). Therefore, both P(3HB) homopolymer and P(3HB-*co*-3HV) copolymer did not find widespread applications as initially expected of them.

Production of poly(4-hydroxybutyrate), a high-value PHA

For the purpose of commercialization, the value of the end product must also be taken into consideration. Indeed, the productivity of PHA copolymers that contain 4HB monomers may be lower compared to P(3HB). However, this type of PHA has interesting properties that make them suitable for biomedical applications, thus becoming a high value product. The poly(4-hydroxybutyrate) homopolymer, P(4HB), is currently the only PHA to be used as FDA-approved monofilament absorbable sutures, and has received CE clearance in Europe as a material for medical devices (Williams *et al*., 2013). Large-scale production of P(4HB) is impeded by several factors. Typically, the incorporation of the 4HB monomer into PHA occurs only when a structurally related substrate is available. Common precursors of P(4HB) biosynthesis include 1,4-butanediol, g-butyrolactone, 4-chlorobutane, or 4HB sodium salt. These precursors are relatively more expensive than P(3HB) carbon sources such as simple sugars, thus increasing the overall cost of production.

Efforts to circumvent these problems have involved development of recombinant strains as well as feeding strategies. Recombinant *E. coli* strains have been engineered to produce the P(4HB) homopolymer, although most of the published studies require the supplementation of both a structurally-unrelated substrate together with the 4HB precursors. The first recombinant *E. coli* capable of incorporating the 4HB monomer into PHA was engineered with the *phaC* gene from *Alcaligenes latus* and an open reading frame, *orfZ* from *Clostridium kluyveri* (Hein *et al*., 1997). Heterologous expression of the PHA synthase and the *orfZ* enabled the strain to produce P(4HB) when glucose and 4-hydroxybutyric acid were supplied simultaneously. However, without the addition of glucose, a copolymer of P(3HB-*co*-4HB) was produced; if only glucose was added without the 4HB precursor, no PHA was accumulated. With the same recombinant *E. coli* strain, an attempt to scale-up production of P(4HB) was performed by Song and colleagues via fed-batch fermentation and feeding of both glucose and 4-hydroxybutyric acid (Song *et al*., 1999). After a 60-h pH-controlled fermentation, the final PHA concentration obtained was 4.4 g/L, culminating in a volumetric productivity of 0.073 g/L·h of P(4HB).

To eliminate the need for structurally-related precursors, production of P(4HB) using glucose as the sole carbon source was successfully established in *E. coli* by co-transformation of plasmids pMCSH5 and pKSSE5.3. The plasmid pMCSH5 contained genes *sucD*, encoding succinate semialdehyde dehydrogenase, and *4hbD*, encoding 4HB dehydrogenase from *C. kluyveri*; plasmid pKSSE5.3 contained *phaC* from *C. necator* and *orfZ* from *C. kluyveri*. Four genes encoding phasins from *C. necator*, which are proteins that bind to the external surface of PHA granules, were inserted along with the plasmids to investigate their effects for improved P(4HB) accumulation. It was found that the insertion of a phasin gene, *phaP1*, led to higher CDW and P(4HB) accumulation, i.e. 5.5 g/L and 35 wt% when fed with 20 g/L glucose as the sole carbon source. Further fed-batch fermentation with this recombinant yielded P(4HB) accumulation of 68 wt%, corresponding to a volumetric productivity of 0.15 g/L·h P(4HB) over a period of 52 h (Zhou *et al*., 2012).

Investigations into factors that influence P(4HB) accumulation in recombinant *E. coli* led to several interesting discoveries. Unlike P(3HB), P(4HB) synthesis was found to be dependent on amino acid concentration and not nitrogen limitation, i.e. P(4HB) was initiated when amino acids were limited. Addition of acetic acid was also found to enhance P(4HB) accumulation when cultivated on glycerol. The rationale for this was related to amino acid limitation within the cells, whereby acetic acid causes a reduction in the intracellular methionine content. An exponential feeding strategy with a mixture of glycerol, acetic acid, and sodium 4HB precursor led to a maximum P(4HB) concentration of 15.0 g/L, with a productivity of 0.205 g/L·h, which is the highest productivity reported for P(4HB) as of now (Le Meur *et al*., 2014).

Ultimately, the production of P(4HB) has to be trade-off between its high value and its low productivity. While there exists a niche market for P(4HB) in the medical industry, its application in other industries are limited. Thus, it is likely that P(4HB) cannot compete in the marketplace as a bulk material, but rather, a specialized product.

Potential of P(3HB-*co*-3HHx) as a commodity plastic

Improvements in polymeric properties can be accomplished by the incorporation of the 3-hydroxyhexanoate (3HHx) monomer to form a copolymer of P(3HB-*co*-3HHx). P(3HB-*co*-3HHx) has lower melting temperature than P(3HB), thus requiring lower processing temperature and consequently lower energy input. Furthermore, it exhibits higher flexibility and impact strength, which broadens its range of application (Doi *et al*., 1995). Indeed, P(3HB-*co*-3HHx) containing 42 mol% of the 3HHx monomer was found to have similar properties to polyethylene with regards to the Young's modulus and elongation to break (Jeon *et al*., 2014).

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In the past, P(3HB-*co*-3HHx) was reportedly synthesized by several *Aeromonas* species with varying 3HHx molar fractions (Kobayashi *et al.*, 1994; Doi *et al.*, 1995). Lee and coworkers reported high-cell-density cultivation of *A. hydrophila* for production of P(3HB-*co*-3HHx) with 17 mol% of the 3HHx monomer (Lee *et al.*, 2000). It was found that the copolymer was only produced when lauric acid and oleic acid were fed and when phosphorus was limiting. Their study also addressed the challenges associated with fed-batch feeding of the aforementioned fatty acids. Specifically, foaming and solidification of the fatty acid at cultivation temperature (30 °C) in the case of lauric acid, and attachment of the oleic acid to the internal parts of the fermentor vessel, making them unavailable for cellular consumption. Several strategies were suggested to improve the fermentation process, including a pH-stat and dissolved oxygen (DO)-stat feeding. The maximum CDW and PHA concentration achieved after 43 h of cultivation was 95.7 g/L and 43.3 g/L respectively, with PHA productivity of 1.01 g/L·h. Large-scale production of P(3HB-*co*-11 mol% 3HHx) by *A. hydrophila* 4AK4 in a 20000-L fermentor has also been demonstrated, with a resulting PHA yield of 25 g/L after 46 h of cultivation (Chen *et al.*, 2001). In this study, 50 g/L glucose was used to support cell growth before the addition of 50 g/L lauric acid to initiate PHA production under phosphorus-limited conditions. However, the PHA productivity was 0.54 g/L·h, which was almost 50% lower than that reported by Lee and coworkers (Lee *et al.*, 2000).

The use of fatty acids in their pure form as a precursor for the 3HHx monomer may be costly. For this reason, researchers have looked towards using plant oils as carbon sources for production of this copolymer. This was exemplified by the study on production of P(3HB-*co*-3HHx) by a recombinant *C. necator* strain using soybean oil as the sole carbon source (Kahar *et al.*, 2004). The recombinant strain was constructed by inserting plasmid pJRDEE32dl3 containing a PHA synthase gene from *Aeromonas caviae* into the *C. necator* mutant, PHB^{-}4 (DSM 541). The CDW obtained from the recombinant strain was 128 to 138 g/L, which was higher than those obtained from wild type *C. necator* H16 (118 to 126 g/L) when grown on the same carbon source. For both strains, the PHA content was more than 70 wt% of the CDW. The recombinant *C. necator* was able to synthesize P(3HB-*co*-3HHx) containing 5 mol% of the 3HHx monomer, while only P(3HB) was synthesized by the wild type strain. After a cultivation period of 96 h in a 10-L fermentor, the maximum volumetric productivity of the recombinant strain was determined to be 1.06 g PHA/L·h, and the yield of PHA per gram of soybean oil was 0.72 g.

Comparisons between batch, extended batch, and fed-batch strategies were investigated by Riedel *et al.* using a recombinant *C. necator* strain (Riedel *et al.*, 2012). The strain used in their study, denoted as Re2058/pCB113, was engineered to incorporate the 3HHx monomer into the PHA polymer chain when palm oil was supplied as the sole carbon source (Budde *et al.*, 2011). In the extended batch fermentation, increased nitrogen source was added, i.e. 4.5 g/L urea compared to 2.25 g/L urea in batch fermentation. Initial palm oil concentration was 40 g/L and an additional 20 g/L was added at hour 32. As a result, the cells were able to accumulate up to 45 wt% PHA at 24 h, which was before nitrogen became limiting. Upon nitrogen limitation, PHA production was maximum, reaching 72 wt% after 96 h. Fed-batch fermentation using ammonium hydroxide to maintain the pH up to 48 h resulted in 59 wt% accumulation of PHA. When nitrogen limitation was incurred after 60 h, a maximum of 70 wt% PHA was achieved. The PHA obtained was a copolymer of P(3HB-*co*-24 mol% 3HHx). In comparison, the use of urea as the nitrogen source in a fed-batch feeding mode gave rise to higher PHA content in the cells. Nitrogen limitation was observed after 63 h, and by the end of fermentation at 96 h, a maximum of 73 wt% P(3HB-*co*-19 mol% 3HHx) was obtained. The volumetric productivity, or space-time-yield as stated in the literature, was 1.1 g PHA/L·h, which is among the highest reported for this copolymer. The study also highlighted the stability of the plasmid in high cell density cultures, which suggested that recombinant strains were suitable for large-scale production of PHA.

Compared to the production of P(3HB), P(3HB-*co*-3HHx) requires the supply of fatty acids. As a result, the use of plant oils are favoured for the production of P(3HB-*co*-3HHx). The use of plant oils as a feedstock has the advantage of being efficiently consumed and converted to PHA by the cells, compared to sugars (Akiyama *et al.*, 2003). The disadvantage is the cost of plant oils, especially when sugars can be obtained from plant waste materials. On the other hand, the biomass must undergo several treatment steps before the sugars can be obtained, which contribute to processing costs and would offset the low cost of the raw material. Global production of plant oils has been steadily increasing over the past decade. In 2005, approximately 118 million metric tonnes was produced. This figure exceeded 175 million metric tonnes in 2015. Palm oil has consistently been the main contributor to global production, followed by soybean, canola, and sunflower oil. Inevitably, concerns may arise over the fluctuations in the prices of these edible oils. However, consumption of these oils worldwide was estimated to be 173 million metric tonnes, which was lower than the total production (Statista, 2016). Thus, prices of oils can be controlled if production continues to outweigh consumption. Furthermore, methods of farming must be closely regulated to meet criteria for sustainability.

EFFICIENCY OF RECOVERY

Conventional methods of recovery

As mentioned earlier, one of the factors that determine the efficiency of PHA production is the recovery process. An efficient recovery approach should yield PHA with high purity and the cost of recovery needs to be as minimal as possible. At the same time, the use of chemicals and solvents should be minimized. Some of the conventional

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methods for PHA extraction include solvent extraction, digestion methods such as chemical and enzymatic digestion, mechanical disruption like bead mill disruption, high pressure homogenization, disruption using ultrasonication, centrifugation and chemical treatment, air-classification method, supercritical fluid method, cell fragility method and dissolved-air flotation method. Here, we revisit the conventional methods of PHA recovery by considering their advantages and disadvantages.

It has long been known that the common methods of PHA recovery revolve around the usage of organic solvents and chemicals. After all, the extraction of polymer with organic solvents is proven to be more superior to the other PHA extraction methods. The solvent extraction process recovers the polymer by improving the cellular membrane permeability and subsequent solubilization of the PHA, hence the PHA was not degraded (Ramsay *et al*., 1994). On top of that, this method was also found to be more effective in preventing Gram-negative bacterial endotoxin contamination of the polymer and hence improve the polymeric quality for biomedical applications particularly (Lee *et al*., 1999). The use of solvent to recover PHA was first described on *Bacillus megaterium* and *Rhodospirillum rubrum*, respectively by Lemoigne, circa 1923-1951, and Baptist in 1967. In this approach, several chlorinated hydrocarbon solvents including chloroform, 1,2-dichloroethane, methylene chloride or cyclic carbonates such as propylene and ethylene carbonates were used (Baptist, 1962). The extraction of PHA from *C. necator* with liquid halogenated solvents (chloroethanes and chloropropanes) were studied by Vanlautem and Gilain (Vanlautem and Gilain, 1982). It was discovered that the best results were achieved with solvents that contain functional carbon atoms with one hydrogen atom and one chlorine atom, at least. Similarly, extracting agents such as diols, acetalized triols, di- or tricarboxylic acid esters and butyrolactone could give good recovery and purity (Traussnig *et al*., 1990).

Besides that, acetic anhydride, tetrahydrofuran ethyl cyanide and tetrahydrofuran methyl cyanide were also used for extracting PHA. Although the recovery was not very high, it could produce high purity after a few downstream processes. It was reported that the purity of P(3HB-*co*-3HV) extracted from *C. necator* biomass with methylene chloride was above 98% after being concentrated by distillation, precipitation on ice-cold methanol and recrystallization (Zinn *et al*., 2003). A recent recovery method, methyl ethyl ketone (MEK)-based extraction was proposed by Yang and colleagues. They reported that MEK was a promising candidate solvent for PHA recovery from bacterial cells such as *C. necator* and *E. coli.* By using this method, more than 90% of polymer recovery was achieved in P(3HB-*co*-3HV). MEK has desirable characteristics such as low viscosity and easy separation by sedimentation and thus reduce the costly and lengthy steps in the extraction process (Yang *et al*., 2015).

Another method which is commonly used is chemical digestion. Surfactants such as anionic sodium dodecyl sulfate (SDS) could disrupt cells by integrating itself into the lipid bilayer membrane and increase the volume of the cell envelope until it is saturated. The cells were lysed and micelles of surfactant and membrane phospholipids were produced, resulting in the release of P(3HB) into the solution surrounded by cellular debris (Ramsay *et al*., 1990). Synthetic palmitoyl carnitine was shown to lyze *C. necator* and *Alcaligenes latus* with the lysis of above 70% and 85%, respectively (Lee *et al*., 1993). It was also shown that in *C. necator*, purity over 95% and a recovery exceeding 90% for P(3HB) was obtained using a SDS/biomass ratio higher than 0.4 (Kim *et al*., 2003). Sodium hypochlorite was also used for differential digestion of non-PHA cellular materials. It was reported that high purity levels of PHA in *C. necator* and recombinant *E. coli* were obtained using this method with 86% and 93%, respectively (Hahn *et al*., 1994). However, sodium hypochlorite could reduce the molecular weight of PHA by 50% (Berger *et al*., 1989). The use of heat pretreatment at 50 °C and a digestion with 4 mL sodium hypochlorite on *C. taiwanensis* 184, resulted 99% of purity and 94% of recovery. Meanwhile in *Pseudomonas putida* KT2442, 99% of purity and 78% of recovery were achieved by using 35 mL of sodium hypochlorite solution (4.1%) (Lu, 2006). A combination of surfactant and hypochlorite as a recovery process of *Azotobacter chroococcum* G-3, resulted in a purity of 98% and recovery of 86.6% was reported by Dong and Sun (Dong and Sun, 2000). The addition of chelate to the surfactant could also increase the release of PHA. Indeed, the recovery of P(3HB) from *C. necator* was as high as 93.3% and a purity of 98.7% was obtained (Chen *et al*., 1999).

As an alternative to solvent extraction, enzymatic digestion method was developed by Zeneca Group PLC (Holmes and Lim, 1990). This method was used to recover medium-chain-length (MCL) PHA from *P. putida* and the purity of almost 93% was reported by Kathiraser and colleagues (Kathiraser *et al*., 2007). A complete lysis of *C. necator* cells upon treatment with lytic enzymes of *Cytophaga* sp. without mechanical processing was discovered by Harrison and coworkers (Harrison *et al*., 1990). Kapritchkoff and colleagues found out that 88.8% P(3HB) purity was obtained by utilizing 2% of bromelain (enzyme mass per biomass) while 90% of polymer purity could be achieved with pancreatin, an enzyme which is three times cheaper than bromelain) (Kapritchkoff *et al*., 2006). Combination of hypochlorite, SDS or alkali treatment, EDTA, heat, with enzymes (Lysozyme, Neutrase, Alcalase, Lecitase) were also tested for the extraction of PHA (De Koning and Witholt, 1997). Nevertheless, enzymes are too expensive for use in a large-scale extraction process although the purity and recovery of PHA are promising.

Mechanical cell disruption treatments have also been used for recovering the intracellular PHAs. A combination method of high pressure homogenizer and SDS was used

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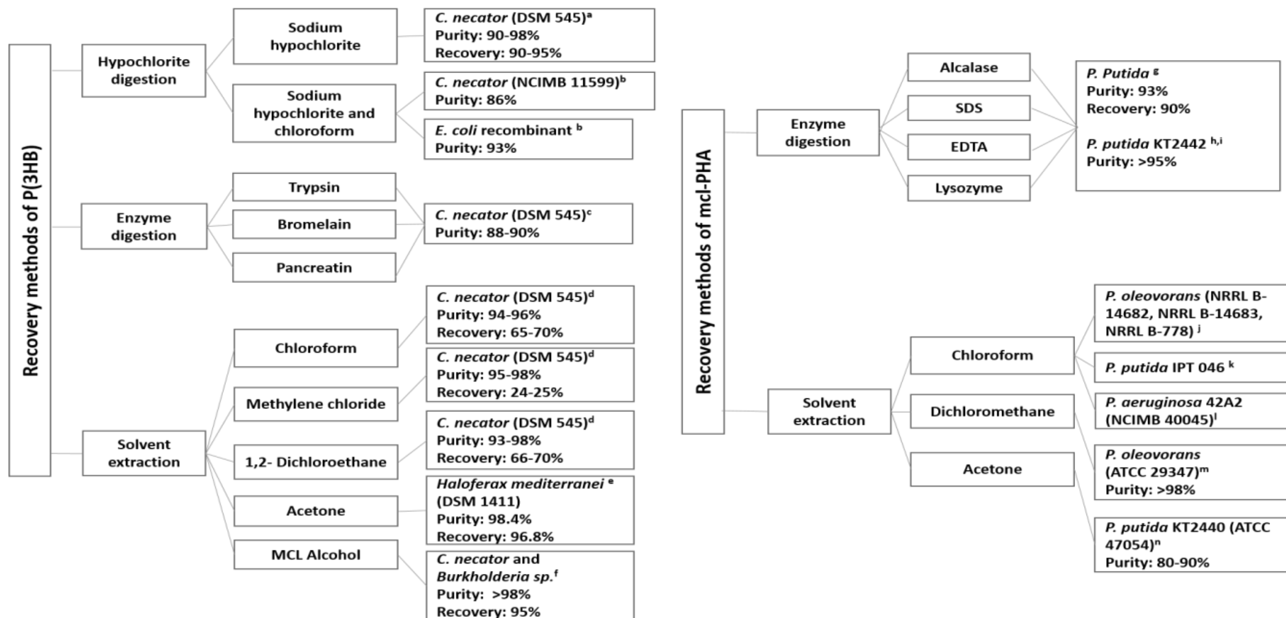

Figure 3: Recovery methods of P(3HB) and mcl-PHA.
[a], Berger *et al*., 1989; [b], Hahn *et al*., 1995; [c], Kapritchkoff *et al*., 2006; [d], Ramsay *et al*., 1994; [e], Koller *et al*., 2013; [f], Nonato *et al*., 2001; [g], Yasotha *et al*., 2006; [h], De Koning and Witholt, 1997; [i], De Koning *et al*., 1997; [j], Ashby *et al*., 2002; [k], Sánchez *et al*., 2003; [l], Fernández *et al*., 2005; [m], Durner *et al*., 2000, [n], Jiang *et al*., 2006.

to recover P(3HB) from *Methylobacterium* sp. V49. By using a 5% (w/v) SDS solution with an operating pressure of 400 kg/cm^2 after two cycles, a maximum yield of 98% and purity of 95% were obtained (Ghatnekar *et al*., 2002). The centrifugation and chemical treatment for efficient removal of non-P(3HB) cell material was investigated by Van Wegen and colleagues (Van Wegen *et al*., 1998). After three centrifugation steps, a P(3HB) recovery of 80% and a purity of 98.5% (w/w) were achieved. Besides that, supercritical fluids were also used as extraction solvent for PHA recovery. Hejazi and coworkers showed that the optimal conditions for cell disruption and recovery of P(3HB) from *C. necator* were when the exposure time is 100 min, at a pressure of 200 atm, a temperature of 40 °C and the use of 0.2 mL of methanol (as a polar modifier). The final recovery achieved was as high as 89% (Hejazi *et al*., 2003). Some examples of the recovery methods for P(3HB) and MCL PHAs are shown in Figure 3.

Apart from that, several methods of PHA extraction and recovery have also been patented. For example, Mantellato and Durao patented their method of extracting and recovering PHAs from cellular biomass under the publication number US 20080193987 A1. This method comprised of the steps of extracting the PHA with non-halogenated solvent that is non-aggressive to the environment and yields PHAs of high purity and high molecular weight. This was an invention suited for industrial scale process in which it minimized the time of exposure of most PHA extracted from the cellular biomass at high temperature, using non-halogenated solvents and thus minimized its degradation and preserved its natural properties especially its molecular weight. The purity as high as 99% and 90% of PHA recovery were achieved (Mantellato and Durao, 2008). A method for recovery and purification of PHAs was patented under the publication number US007514525B2. The patent suggested a method to recover, purify and modify PHA biopolymer solids from PHA-containing cells by solubilizing the non-PHA cell mass (NPCM) in an acidic solution, leaving a suspension of partially crystallized PHA granules, followed by adjustment of pH to 7-11 and separating the PHA solids from the dissolved NPCM, suspending the PHA solids in bleach solution and finally drying of the PHA solids. More than 95% of original PHA in cell mass was recovered and 97% of purity was achieved (Yu, 2009).

Escalona and colleagues proposed the method for extraction of PHAs from halophilic bacteria and halophiles. The concept was based on the weakness of the cell envelopes of these microorganisms when they are exposed to low concentration of salts in which the cells of halophilic bacteria will lyse or rupture and thus releasing all the cell components into the medium. As the PHA granules are of substantial size and density, they can be recovered from the cell suspension once lysed, by centrifugation at low speed, sedimentation or filtration. This procedure was patented with the number US 005536419A (Escalona *et al*., 1996).

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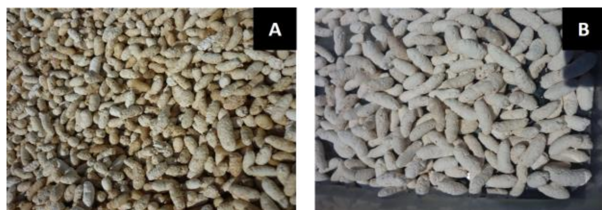

Figure 4: The appearance of the faeces of rats before washing (A) and after washing (B). The rats were fed with freeze-dried *C. necator* cells containing PHA granules.

Biological recovery as an alternative method

Conventional methods for PHA recovery are effective in terms of yield and purity. Nevertheless, they also pose a threat to the environment. The use of surfactants generates waste to the water system while the use of chemicals and solvents are hazardous to the user and to the environment. In order to recover PHA in a safer manner, biological recovery using animals could be a possible alternative to the current PHA extraction method. A newly developed biological recovery process using laboratory rats was proposed by Kunasundari and coworkers (Kunasundari *et al.*, 2013). Their study reported that *C. necator* H16 cells could be fed as a protein source to certain animals. The PHA granules cannot be digested and therefore are excreted in the form of white fecal pellets containing almost 90 wt% PHA. Simple rinsing of the fecal pellets with water resulted in PHA purity of more than 95%. The difference between washed and unwashed fecal pellets is shown in Figure 4. This method enabled the extraction and purification of kilogram quantities of PHA in the laboratory for the development of controlled release fertilizer (unpublished results from the authors' laboratory), which is impossible if one were to use chloroform.

This biological recovery method has also been patented under the publication number WO2010134798 A1. The invention provided the method for extracting and purifying PHA and other polyester granules by natural and environmentally friendly approach, which applied the concept of digestive system of suitable animals. Since this process does not require the addition of any chemicals or solvents for the recovery of polyesters, the PHAs obtained are 100% bio-based. It was proposed that the digestive system of the suitable animals is capable of hydrolyzing the biomass material with the various enzymes available in the digestive tract. The compounds that could not be hydrolyzed such as PHA will be excreted as fecal pellets (Sudesh, 2010).

With this breakthrough in PHA recovery, it is possible to recover PHA using small animals. Indeed, some of the industrial product have been using animal as a source of recovery machinery. For example, the world's most expensive coffee, Black Ivory Coffee is now being produced in Thailand's Golden Triangle by feeding elephants with coffee beans. Their faeces containing the coffee beans are then collected and processed (Black Ivory Coffee Company Limited, 2012). Similarly, *Kopi Luwak* or Civet coffee has been produced in a similar manner. The coffee includes digested coffee cherries, which are consumed by the Asian palm civet (*Paradoxurus hermaphroditus*) and excreted in their faeces. A study has shown that the processing through the civet's gastro-intestinal track significantly modifies the coffee bean (Marcone, 2004). It is said that the digestive enzymes in these animals have added flavor to the coffee beans.

In view of PHA recovery, apart from using conventional methods, which are expensive and not environmentally-friendly, their drawbacks could be compensated with the use of living organisms that could purify the PHA in their intestine. The use of suitable animals as a source of biological recovery could complement the use of organic solvents and surfactants. The faeces produced by these living organisms still require a series of washing depending on the percentage of PHA in the faeces. Thus, a combination of conventional method and biological recovery should go hand in hand. However, if cells contain high amount of PHA, it is possible that it might not need to go through the washing process. Nevertheless, this is dependent on the final application of the polymer. Biologically recovered PHA may be applicable in products where high level of purity is not necessary such as for the making of biodegradable mulching films and slow or controlled release fertilizers. Advantages and disadvantages of using conventional methods and biological recovery methods are summarized in Figure 5.

Criteria for large scale biological recovery processes, potential hazard and precaution

As biological recovery processes involve the use of living organism, it is important that the procedure should fulfill the animal ethic requirement. The process should also be hazard free and do not pose any health issue to the user. Thus, precaution should be taken into account when dealing with animals. Some of the potential hazard that could arise when using animals is the pathogen and disease-related factor. It will be a hassle if they are infected by certain diseases, as this will affect the production and recovery of PHA. The selection of suitable animal as recovery machinery is very crucial as it will determine the success of the PHA recovery in the long run. The animal chosen should be healthy, could ingest PHA cells without any complications, and has commercial value when produced in large scale.

This approach for recovery of PHA is still considered very new and thus a lot of experiments need to be done in order to ensure that this method is safe and reproducible. Depending on which animals are used for the recovery process, their life cycle and diet need to be understood completely. The selection and use of appropriate living organism as recovery machinery will surely help to increase the efficiency of PHA recovery. An efficient recovery process should meet the criteria of high yield, high purity, less time in processing and lower cost of production.

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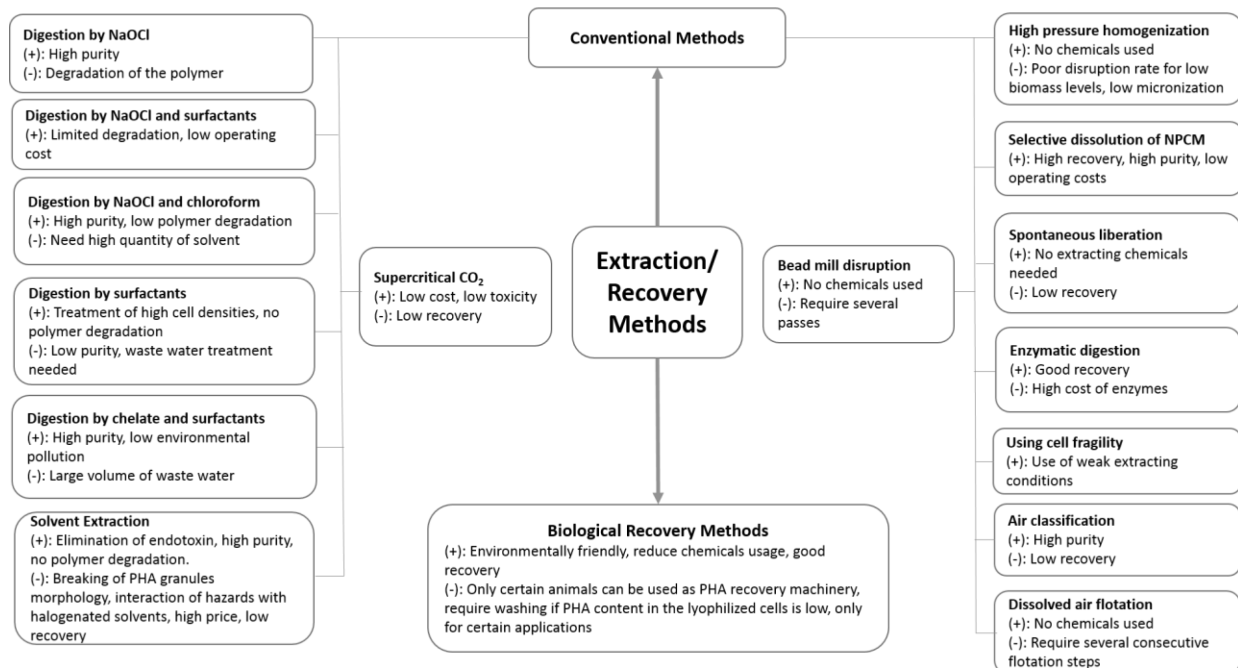

Figure 5: The advantages and disadvantages of conventional and biological recovery methods of PHA from bacterial cells. +, indicates advantages; -, indicates disadvantages.

CONCLUSION

In the near future, it is expected that plastic pollution would reach a critical stage whereby renewable plastic materials would no longer be an alternative, but an essential. This is despite the fact that a certain type of bacterium has been found in Japan to be able to hydrolyze polyethylene terephthalate (PET) (Yoshida *et al.*, 2016). There is no doubt that great strides have been made in the field of PHA research, with an emphasis on improving the efficiency of production. The major parameters that determine efficiency have been proposed in this review, i.e. time, cost, productivity, recovery, stability, and commercial viability. However, as discussed in this review, it is difficult to develop a single process that is optimized for all parameters. Nevertheless, the rapid and intensive efforts towards efficient production of PHA have witnessed positive returns, albeit on a smaller scale, and should be taken as an encouragement to continue the pursuit of PHA commercialization.

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