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Isolation and recovery of microbial polyhydroxyalkanoates

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Abstract. The deleterious environmental impacts caused by plastic wastes have attracted worldwide concern. The bio-based and biodegradable polyhydroxyalkanoate (PHA) appears to be one of the potential candidates to replace some conventional plastics. However, high production cost of PHAs has limited their market penetration. The major cost absorbing factors are the upstream fermentation processes and the downstream PHA recovery technologies. The latter significantly affects the overall process economics. Various recovery technologies have been proposed and studied in small scales in the laboratory as well as in industrial scales. These include solvent extraction, chemical digestion, enzymatic treatment and mechanical disruption, supercritical fluid disruption, flotation techniques, use of gamma irradiation and aqueous two-phase system. This paper reviews all the recovery methods known to date and compares their efficiency and the quality of the resulting PHA. Some of the large-scale production of PHA and the strategies employed to reduce the production cost are also discussed.

Keywords: *biodegradable polymers, polyhydroxyalkanoate, recovery, microorganism*

1. Introduction

The versatility of plastic materials in terms of mechanical properties and durability has been manipulated by mankind to enhance quality of life without realizing they have become increasingly ubiquitous. The world's plastics production was estimated to be 260 million tonnes in 2007 [1]. It is clear from this figure that the long term deleterious environmental impacts caused by plastics were entirely overlooked and this in turn poses greater difficulties for plastic waste disposal. Therefore, the development and use of biodegradable plastics is gaining more serious attention. The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoates (PHA) and polylactic acid (PLA) [2]. PHA is attractive because of its biodegradability and physical properties that closely resemble some conventional plastics such as polypropylene (PP) and low-density polyethylene (LDPE) [3]. In addition, because of the diverse types of monomers (about 150 different structures), it is possible to produce PHA copolymers having a wide range of properties. The various PHA monomers can be classified based on the number of carbon atoms as short-chain length PHA (scl-PHA), medium-chain length PHA (mcl-PHA) and long-chain length PHA (lcl-PHA). Scl-PHA refers to PHA comprised of monomers having 5 or less carbon atoms. These include 3-hydroxybutyrate and 3-hydroxyvalerate. The mcl-PHA is comprised of monomers having 6 to 14 carbon atoms. These include 3-hydroxyhexanoate, 3-octanoate and 3-hydroxydecanoate. The lcl-PHA, which is uncommon and least studied, consists of monomers with more than 14 carbon atoms. Recently, it has also been made possible to synthesize a new type of PHA containing lactide as a co-monomer [4–6]. All these developments indicate that PHA may become the preferred next generation bioplastic. However, to date the market penetration of PHA is still scarce. This is mainly due to

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its high production cost. The main reasons behind the economic disadvantages are the costly fermentation and purification technologies. The latter significantly affects the overall process economics. Much work has been carried out to lower the PHA production cost by the use of effective and inexpensive carbon source and genetically engineered microorganisms. Sugars have been shown to be an effective feedstock for PHA production in Brazil, especially when the PHA production is integrated to the sugarcane-processing factory [7]. On the other hand, it has been demonstrated that vegetable oils are also potential feedstock for PHA production [8–12] in countries like Malaysia where palm oil is produced in very large scales. High levels of PHA accumulation have been achieved using crude palm kernel oil. The yield of PHA from vegetable oils is at least two times the yield of PHA from sugars [13]. However, the real cost associated with PHA would only diminish with the development of a cheaper and environmentally friendly PHA recovery method. This article reviews the currently known methods for the recovery of PHA. Some of the large-scale production of PHA and the strategies employed to reduce the production cost are also discussed.

2. PHA biosynthesis

PHA is a lipid-like compound synthesized by many microorganisms as a form of storage material. Upon

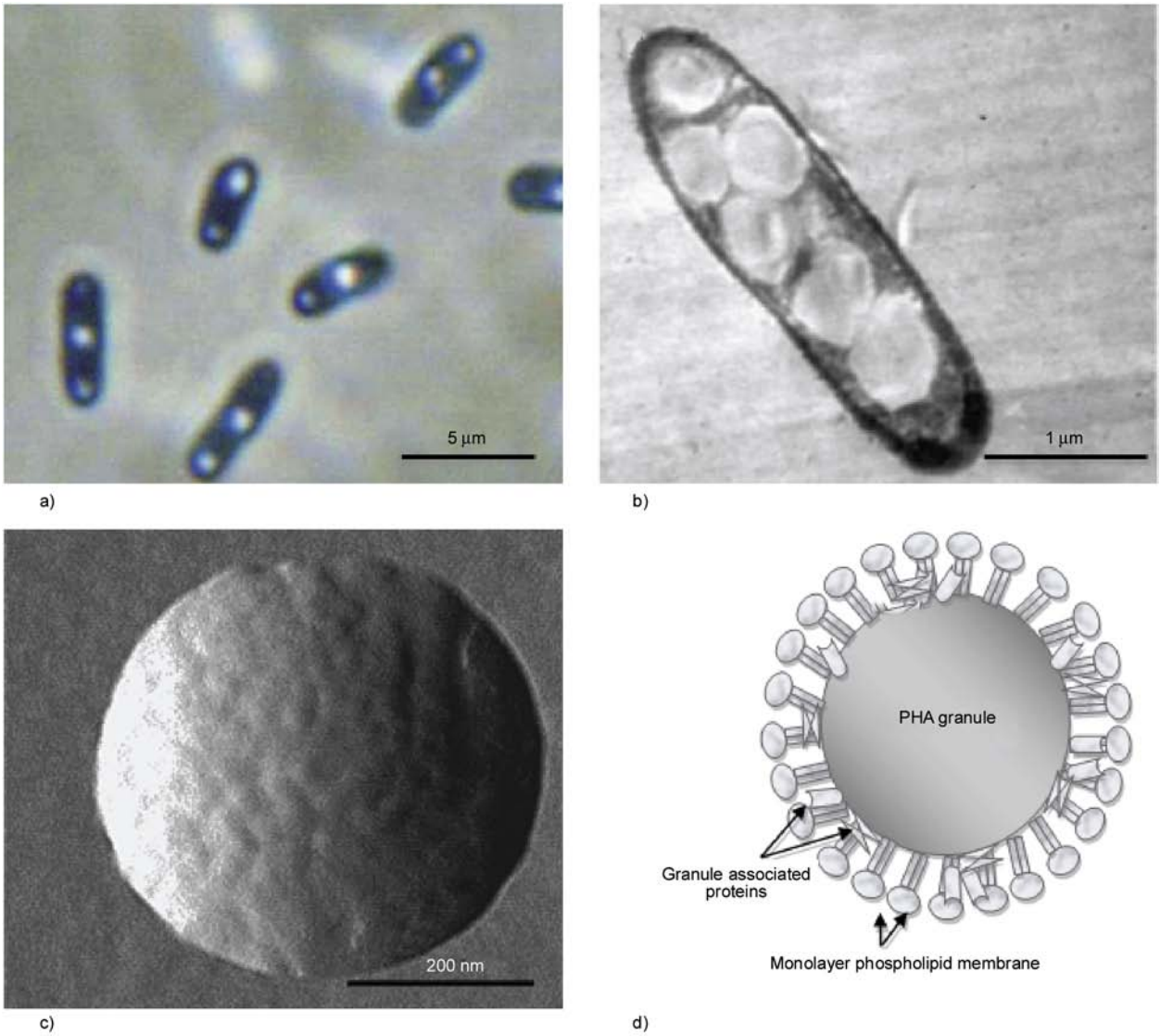

Figure 1. Morphology of PHA granules in the bacterial cells observed under (a) Phase contrast and (b) Transmission Electron Microscope. (c) Atomic force microscope deflection image showing the presence of globular particles on the granule surface. (d) A model representing the native PHA granule with a protein monolayer on the surface (Not drawn according to actual scale)

synthesis, PHA is accumulated in the form of granules in the bacterial cell cytoplasm. The average size of the PHA granules is approximately 0.2–0.5 μm (Figure 1). Figure 1 shows the morphology of PHA granules when observed using various microscopy techniques. Atomic force microscopy analysis has shown the presence of a protein monolayer on the surface of PHA granules [14–15]. In order to recover the PHA granules, it is necessary to rupture the bacterial cell and remove the protein layer that coats the PHA granules. Alternatively, the PHA has to be selectively dissolved in a suitable solvent. Formation of PHA granules is dependent on the presence of suitable metabolic pathways. Figure 2 shows a simplified metabolic pathway for the biosynthesis of poly(3-hydroxybutyrate) [P(3HB)], which is the most common type of PHA. The biosynthesis of P(3HB) is initiated by the condensation of two acetyl-CoA molecules by β-ketothiolase (PhaA) to form acetoacetyl-CoA. Subsequently, NADPH-dependent acetoacetyl-CoA reductase (PhaB) catalyzes the reduction of acetoacetyl-CoA to the (R)-isomer of 3-hydroxybutyryl-CoA which is then polymerized into P(3HB) by the PHA synthase (PhaC) [3]. To date, *Cupriavidus necator* (formerly known as *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus*) is the most extensively studied microorganism for the cost-effective production of PHA. Numerous other strains such as *Bacillus cereus* SPV, *Sinorhizobium meliloti*, *Azotobacter chroococcum* G-3, *Pseudomonas putida* KT2440 and *Metylobacterium* sp V49 also gaining attention for the PHA production. Besides wild-type strains, recombinant strains are also being developed. Various types of recombinant *Escherichia coli* strains are able to synthesize PHA to high intracellular level and some are amenable to genetically mediated lysis system to facilitate the release of the PHA granules [16]. Compared to the extensive research on the biosynthesis of PHA, research on the downstream processing of PHA is scarce. Table 1 shows the various extraction methods that have been reported. The following section will describe all these methods and finally compare their advantages and disadvantages (Table 2). This study is important to choose a suitable method that could effectively isolate PHA from the microbial cells.

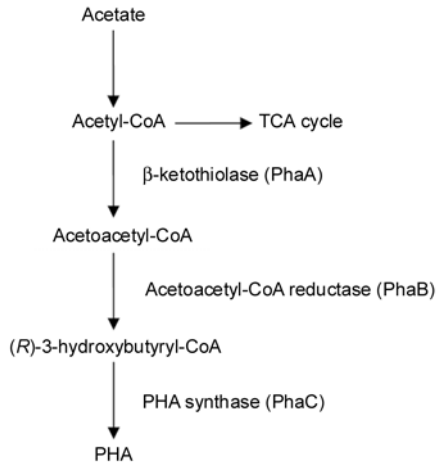

Figure 2. The most studied metabolic pathway for the biosynthesis of polyhydroxyalkanoates (PHA)

3. Recovery techniques for the isolation and purification of PHA from microbial cells

3.1. Solvent extraction

Solvent extraction is the most extensively adopted method to recover PHA from the cell biomass (Table 1). This method is also used routinely in the laboratory because of its simplicity and rapidity. Two main steps are involved, first is the modification of cell membrane permeability thus allowing release and solubilization of PHA. This is then followed by non-solvent precipitation [17]. Extraction of PHA with solvents such as chlorinated hydrocarbons, i.e. chloroform, 1,2-dichloroethane [18] or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate [19] is common. Lower chain ketone such as acetone is the most prominent solvent especially for the extraction of mcl-PHA [20]. Precipitation of PHA is commonly induced by non-solvent such as methanol and ethanol [18]. Solvent extraction has undoubted advantages over the other extraction methods of PHA in terms of efficiency. This method is also able to remove bacterial endotoxin and causes negligible degradation to the polymers [17]. So, it is possible to obtain very pure PHA with high molecular weights. Unfortunately, large-scale application of solvent extraction is generally viewed as a method that is not environmentally friendly. In addition, several other factors discourage the use of solvents such as high capital and operational costs. Another problem is the high viscosity of the extracted polymer solution when the P(3HB) concentration exceeds 5% (w/v) (Table 2). The viscosity of the solution interferes with cell debris removal resulting in lengthy separation process. Besides, there is a possibility that solvent

Table 1. Various PHA recovery methods that have been reported

Extraction method	Comments	Strain	Results	Reference
Solvent extraction	Chloroform	*Bacillus cereus* SPV	Purity: 92%; Yield: 31%	Valappil *et al.* [31]
	Chloroform	*Cupriavidus necator* DSM 545	Purity: 95%; Yield: 96%	Fiorese *et al.* [25]
	1,2-Propylene carbonate	*C. necator* DSM 545	Purity: 84%; Yield:95%	Fiorese *et al.* [25]
	Acetone-water process		Yield: 80–85%	Narasimhan *et al.* [65]
	Methyl *tert*-butyl ether	*Pseudomonas putida* KT2440	Yield: 15–17.5 wt%	Wampfler *et al.* [26]
	Methylene chloride	*C. necator*	Purity: 98%	Zinn *et al.* [66]
	Non halogenated solvents-isoamy propionate, propyl butyrate, isoamyl valerat etc.	*C. necator*		Mantelatto and Durao [67]
	Acetone, room temperature	*P. putida* GPo1	Yield: 94%	Elbahloul and Steinbüchel [64]
Digestion method				
Surfactant	SDS	Recombinant *Escherichia coli*	Purity: 99%; Yield:89%	Choi and Lee [54]
	Palmitoyl carnitine	*C. necator*, *Alcaligenes latus*	Degree of lysis: 56–78%	Lee *et al.* [68]
Sodium hypochlorite	Sodium hypochlorite	*C. necator*, Recombinant *E. coli*	Purity: 86%; Purity: 93%	Hahn *et al.* [69]
	Sodium hypochlorite	*C. necator* DSM 545	Purity: 98%	Berger *et al.* [70]
Surfactant-sodium hypochlorite	SDS-Sodium hypochlorite	*Azotobacter chroococcum* G-3	Purity: 98%; Yield: 87%	Dong and Sun [28]
	Triton X-100-sodium hypochlorite	*C. necator* DSM 545	Purity: 98%	Ramsay *et al.* [27]
Surfactant-Chelate	Triton X-100-EDTA	*Sinorhizobium meliloti*	Purity: 68%	Lakshman and Shamala [34]
	Betaine-EDTA disodium salt	*C. necator* DSM 545	Purity: >96%; Yield: 90%	Chen *et al.* [33]
Dispersion of sodium hypochlorite and chloroform	Chloroform- sodium hypochlorite	*B. cereus* SPV	Purity: 95%; Yield: 30%	Valappil *et al.* [31]
	Chloroform- sodium hypochlorite	*C. necator*, Recombinant *E. coli*	Purity: >98%	Hahn *et al.* [69]
Selective dissolution by protons	Sulfuric acid	*C. necator*	Purity: >97%; Yield: > 95%	Yu and Chen [21]
Enzymatic digestion	*Microbispora* sp culture-chloroform	*S. meliloti*	Purity: 94%	Lakshman and Shamala [34]
	Enzyme combined with SDS-EDTA	*P. putida*	Purity: 93%	Kathiraser *et al.* [71]
	Bromelain; pancreatin	*C. necator*	Purity: 89%; Purity: 90%	Kapritchkoff *et al.* [39]
Mechanical disruption	Bead mill	*A. latus*		Tamer *et al.* [23]
	High pressure homogenization	*A. latus*		Tamer *et al.* [23]
	SDS-High pressure homogenization	*Metylobacterium* sp V49	Purity: 95%; Yield: 98%	Ghatnekar *et al.* [48]
	Sonication	Bacil*lus flexus*	Purity: 92%; Yield: 20%	Divyashree *et al.* [37]
Supercritical fluid	SC-CO_2	*C. necator*	Yield: 89%	Hejazi *et al.* [49]
Cell fragility	Chloroform	*B. flexus*	Yield: 43%	Divyashree and Shamala [56]
	Sodium hypochlorite	*B. flexus*	Yield: 50%	Divyashree and Shamala [56]
	Alkaline hydrolysis	*B. flexus*	Yield: 50%	Divyashree and Shamala [56]
Self flotation of cell debris	Chloroform	*Zobellella denitrificans* MW1	Purity: 98%; Yield: 85%	Ibrahim and Steinbüchel [57]
Dissolved air flotation	Enzymatic hydrolysis, sonification, flotation	*P. putida*	Purity: 86%	van Hee *et al.* [58]
Aqueous two phase system	*Microbispora* sp culture-ATPS	*B. flexus*	Purity: 95%; Yield: 50%	Divyashree *et al.* [37]
Gamma irradiation	Radiation-chloroform	*B. flexus*	Yield: 45–54%	Divyashree and Shamala [61]
Air classification		*E. coli* *C. necator*	Purity: 97%; Yield: 90% Purity: 95%; Yield: 85%	Noda [72] Noda [72]
Spontaneous liberation		*E. coli*	Autolysis of 80%	Jung *et al.* [73]

Table 2. Comparison of the advantages and disadvantages of various PHA extraction methods

Recovery method	Advantages	Disadvantages
Solvent extraction	Removal of endotoxin Useful for medical applications High purity Negligible/limited degradation to the polymer Higher molecular weight	Not environmentally friendly Consumption of large volume of toxic and volatile solvents High capital and operation cost Difficulty in extracting PHA from solution containing more than 5% (w/v) P(3HB) Lengthy process Native order of polymer chains in PHA granules might be disrupted
Chemical Digestion Surfactant	Extracted PHA retains original molecular weight Native order of polymer chains in PHA granules is retained	Low purity of PHA Treatment required to remove surfactant from wastewater
Sodium hypochlorite	Higher purity of PHA can be obtained	Severe reduction in molecular weight of the extracted PHA
Sequential surfactant-hypochlorite	High quality of PHA Rapid recovery and simple process Retain native order of polymer chains in PHA granules Lower operating cost compared to solvent extraction	Combined cost of surfactant and sodium hypochlorite Wastewater treatment required to remove residual surfactant and sodium hypochlorite
Dispersion sodium hypochlorite and solvent extraction	High purity of PHA Reduced viscosity of solvent phase due to digestion of non-polymer cellular material (NPCM) by sodium hypochlorite	Not environmentally friendly Consumption of large volume of toxic and volatile solvents Higher recovery cost
Surfactant-chelate	Convenient operation High quality of product	Produce large volume of wastewater
Selective dissolution of NPCM by protons	Low operating cost Higher recovery yield	Severe reduction in molecular weight if the process parameters are not controlled stringently
Enzymatic digestion	Mild operation conditions Good recovery with good quality	Complex process High cost of enzymes
Bead mill	No chemicals used Less contamination Not susceptible to blockages No micronization of PHA granules	Require several passes Long processing time Various process parameters have to be controlled precisely
High pressure homogenization	No chemicals used Less contamination	Severe micronization of PHA granules Depends on both process and microbial physiological parameters Possible for thermal degradation of desired products Formation of fine cellular debris that interfere with downstream processing
Supercritical fluid (SCF)	Simple Inexpensive Rapid Environmentally friendly	Dependent on process parameters Frequent need for clean up Difficulties in extracting polar analytes Difficulties in dealing with natural samples
Cell fragility	Simple recovery method Mild extraction conditions	Need to balance cell wall softening and cell wall integrity
Self-flotation	Cost effective as additional steps could be avoided	Consumption of large volume of toxic and volatile solvents
Dissolved-air flotation	No chemicals used Less contamination	Requires several consecutive flotation steps
Aqueous two phase system (ATPS)	Short processing time Low material cost Low energy consumption Good resolution High yield and a relatively high capacity	Dependent on process parameters Issue of robustness and reproducibility Absence of commercial kits to evaluate ATPS at bench scale Poor understanding of the mechanism
Gamma irradiation	Retention of solvent solubility due to the low degree of cross-linking No chemicals used/Less contamination	Length of irradiation time High initial investment cost

extraction may disrupt the unique nascent state of the P(3HB) granules that maybe useful in certain applications. In case of accidents, the potential release of a large amount of highly toxic and volatile solvents to the environment is also of great concern [17, 21–23]. Therefore, 1,2-propylene carbonate has been proposed as an alternative to halogenated solvents in the recovery process of PHA [24–25]. Higher boiling point (240°C) of 1,2-propylene carbonate prevents the evaporation to the environment

at lower temperatures and allows its reusability for several cycles of purification. This could reduce the solvent consumption and therefore it is viewed as economically advantageous. Besides, 1,2-propylene carbonate is considered safe due to its low toxicity. It is widely used in many applications including cosmetics [24]. Fiorese *et al.* [25] reported a maximum PHA yield of 95% and a purity of 84% when extracted from the *C. necator* cells at 130°C for 30 min without involving any pretreatment. This is comparable to the values obtained from chloroform extraction (94% yield and 98% purity). For the extraction of mcl-PHA, methyl *tert*-butyl ether (MTBE) has been evaluated and the extractability was found to be promising as in the case with chlorinated-solvents [26]. Wampfler *et al*. [26] claimed that MTBE would have lower environmental impact if the recovery of PHA as well as the production and recycling of MTBE could be carried out in closed facilities.

3.2. Digestion methods

While solvent extraction techniques involve the solubilization of the PHA granules, digestion methods involve the solubilization of the cellular materials surrounding the PHA granules. Digestion methods are well established approaches developed as an alternative to solvent extraction and can be classified into either chemical digestion or enzymatic digestion. Because of the ready availability of various chemicals with known properties many studies have been directed towards the development of chemical digestion methods compared to enzymatic digestion.

3.2.1. Chemical digestion

Various chemical digestion methods have been evaluated for the recovery PHA from cellular biomass (Table 1). The approach is based on the solubilization of non-PHA cellular mass (NPCM) and mainly utilizes sodium hypochlorite or surfactants. The important features of sodium hypochlorite such as strong oxidizing properties and non-selectivity can be manipulated to digest NPCM and facilitate PHA recovery [21]. A range of surfactants has been evaluated such as sodium dodecyl sulfate (SDS), Triton X-100, palmitoyl carnitine, betaine and among them, SDS showed good performance. However, the quality of PHA obtained using either surfactant or sodium hypochlorite alone was not good enough (Table 2). Therefore, a combination of surfactant-sodium hypochlorite was used [22]. Isolation of PHA granules by surfactant digestion exhibited lower degree of purity but had slightly higher molecular weight than sodium hypochlorite digestion (Table 2). In contrast, PHA of higher purity was obtained using sodium hypochlorite but with severe degradation of molecular weight up to 50% [27]. Sequential surfactant-hypochlorite treatment promoted better and rapid recovery of PHA [27–28] and resulted in 50% reduction of overall cost when compared to solvent extraction [22]. Yet, the low operating cost [17] and technical simplicity of this process are not complemented by the complex and unresolved problems caused by surfactant in wastewater treatment and relatively high cost of chemical agents such as SDS and sodium hypochlorite (Table 2) [22]. Hahn and co-researchers established a separation process that took advantage of both differential digestion and solvent extraction [29–30]. The hydrophobicity of P(3HB) and hydrophilicity of lyophilized cells accounted for the development of dispersions of a sodium hypochlorite solution and chloroform. A study by Valappil *et al.* [31] described that the high molecular weight of P(3HB) could be retained by using this method. The main limitation is the use of large quantity of solvent that would raise the recovery cost (Table 2). Surfactant-chelate digestion system was also explored to improve cell disruption and to increase the rate of PHA release [32]. Use of recycled wastewater was proposed later as this system produced large volume of wastewater [33]. Surfactant-chelate digestion (Triton X-100 and ethylenediaminetetraacetic acid [EDTA]) could isolate PHA with 90% purity from enzymatically hydrolyzed cells of *S. meliloti* [34]. Another method is the selective dissolution of non-PHA cell mass by protons to enhance PHA recovery [21–22]. This method is comprised of few steps, but the most important is the solubilization of NPCM in an acidic solution to release partially crystallized PHA granules and later subjecting the suspension to decolorization in a bleaching solution. This method was claimed to lower the recovery cost by using cheaper chemicals with higher recovery efficiency. However, the process parameters have to be controlled stringently if the molecular weight is to be maintained at a minimum of 50% the original molecular

weight. The P(3HB) granules recovered by this method was reported to be highly crystalline.

3.2.2. Enzymatic digestion

Recovery process of PHA using enzymatic digestion involves a rather complex procedure (Table 1). Solubilization of cell components other than PHA typically consists of heat treatment, enzymatic hydrolysis and surfactant washing [35]. To date, various types of enzymes, especially proteases, have been evaluated for their efficiency in causing cell lysis [36]. Lakshman and Shamala [34] used *Microbispora* sp. culture, which was identified to secrete protease, in the fermented broth of *S. meliloti* containing 50% of PHA to induce hydrolysis. The culture was introduced to the thermally (80°C for 10 min) inactivated biomass of *S. meliloti* and incubated for 72 h. The *S. meliloti* cells were hydrolyzed by the protease resulting in the release of the intracellular components together with the PHA granules. The culture containing the lysed cells was then subjected to a simple filtration process and PHA of 94% purity was recovered using chloroform extraction. In contrast, PHA with only 66% purity was isolated from the undigested cells by using chloroform extraction. Similar study was conducted recently with a different strain by Divyashree *et al*. [37]. *Microbispora* sp. culture mixed with *Bacillus flexus* and subjected to separation by aqueous two phase system (ATPS) resulted in PHA with 92% purity. The enzyme technique is attractive because of its mild operation conditions (Table 2) [38–39]. Because enzymes are very specific with respect to the reactions they catalyze, recovery of PHA with good quality could be expected. Nevertheless, the high cost of enzymes and complexity of the recovery process outweigh its advantages [39].

3.3. Mechanical disruption

Mechanical cell disruption is widely used to liberate intracellular protein [40]. The concept has been tested to recover PHA from bacterial cells [23]. Among the various mechanical disruption methods, bead milling and high-pressure homogenization dominate the large scale cell disruption in pharmaceutical and biotechnology industries [41]. Unlike other recovery methods, mechanical disruption is favored mainly due to economic advantageous and because it causes mild damage to the products [23]. Mechanical disruption of cells does not involve any chemicals so it minimizes environmental pollution [17] and contamination to the products [23]. In general, the drawbacks of mechanical disruption method are, high capital investment cost, long processing time and difficulty in scaling up [42–43].

3.3.1. Bead mill

The principle of bead mills is based on the shearing action and energy transfer from beads to cells in the contact zones (Table 1). The key parameters that affect the disruption process are the bead loading and bead diameter [38]. Tamer *et al*. [23] reported that with bead diameter of 512 μm and 2800 rpm agitation speed, complete disruption of the *Alcaligenes latus* cells was achieved after eight passes when the loading was 85% compared to loading of 75% that required more than 16 passes to release most of the cellular protein. The extent of cell disruption also depends on numerous parameters such as residence time distribution (RTD), shear forces, type of microorganisms, cell concentration, feed rate of the suspension, agitator speed, geometry of the grinding chamber and design of the stirrer [44]. Bead mills disruption was recommended for PHA recovery as it requires less power supply, not susceptible to blockages and different diameter of beads did not significantly affect the disruption rate although micronization of P(3HB) is possible with smaller bead size [23]. The major concern is that large number of factors has to be considered to establish a good bead mill disruption system (Table 2) [44].

3.3.2. High pressure homogenization

With high pressure homogenization, disruption of cell suspension occurs under high pressure through an adjustable, restricted orifice discharge valve (Table 1) [45]. Process parameters such as operating pressure, number of passes, suspension temperature and homogenizer valve design must be carefully scrutinized for efficient disruption [46–47]. It was reported that less efficient recovery of P(3HB) from *A. latus* was obtained with homogenizer compared to bead mill disruption due to severe micronization [23]. Nevertheless, P(3HB) with 95% purity and 98% yield was recovered from 5% (w/v) SDS pretreated *Methylobacterium* sp V49 cells subjected to homogenization at an operating pressure 400 $kg \cdot cm^{-2}$ after two cycles [48]. Process parame-

ters are not the only factors that influence the cell disruption but microbial physiological parameters, namely type and growth phase of the microorganisms as well as cell concentration also affect the disruption efficiency (Table 2). Generally, Gram-positive bacteria are more difficult to be disrupted compared to Gram-negative bacteria [47]. Among the drawbacks associated with high pressure homogenization include the possibility of thermal degradation of desired products [48] and formation of fine cellular debris that would interfere with the further downstream processing of PHA granules [46].

3.4. Supercritical fluid (SCF)

Supercritical fluids (SCF) have emerged as a potential extraction technique in the areas of PHA recovery (Table 1) [49–51]. The unique physicochemical properties of SCF such as high density and low viscosities proposed them as suitable extraction solvents [49]. The advantages offered by SCF have led to its popularity (Table 3). Supercritical-carbon dioxide (SC–CO_2) is the most predominantly used SCF due to its low toxicity and reactivity, moderate critical temperature and pressure (31°C and 73 atm), availability, low cost, and non-flammability [49]. By using this method, P(3HB) recovery of 89% from *C. necator* at 200 atm, 40°C and 0.2 ml of methanol was reported [49]. Besides, there is a patent literature which describes on the purification of biopolymers such as rubber from plant material using SCF [52]. Although, the recovery obtained are comparable to other methods, it has to be highlighted that SC–CO_2 efficiency in bacterial cell disruption is very much dependent on the process parameters such as operating pressure, temperature, type of modifier as well as culture cultivation time (Table 2) [53]. High temperature and pressure significantly influence the physiological properties of cell membrane that prevent the biopolymer from being extracted. The addition of modifier plays an important role in increasing the polarity of the CO_2 therefore suitable modifier should be selected to enhance the cell wall permeability. Matured cells are difficult to be disrupted compared to those in early-exponential phase as new proteins would be synthesized increasing the cell resistance to disruption. In order to make the process more economically viable, Khosravi-Darani *et al*. [53] investigated on a series of pretreatment to improve the SC–CO_2 disruption. They found that with 1% (v/v) toluene as a modifier, 200 bar of pressure, 30°C temperature and two times SC–CO_2 pressure release, up to 81% P(3HB) recovery could be achieved by using wet cells of *C. necator*. To further improve the purity of the P(3HB), chemical pretreatment with 0.4% (w/w) sodium hydroxide (NaOH) was employed. The proposed recovery process of PHA is more economical as the costly freeze drying step could be avoided.

Table 3. Summary of supercritical fluids (SCF) cell disruption

Extraction method	Advantages	Disadvantages	References
Supercritical fluid (SCF)	Simple, inexpensive Rapid Environmentally friendly Fluid solvation power Minimizes equipment and labor needs, contamination and loss of yield Solution for drastic problems related to • non-thermal cell inactivation • enzyme inactivation • permeabilization • extraction of fermentation products	Frequent need for clean up Difficulties in • extracting polar analytes • measurement and prediction of biomolecules solubility at varying pressure and temperature for process optimization • dealing with natural samples	Luque de Castro and Jiménez-Carmona [74] Khosravi-Darani *et al.* [53] Cornish *et al.* [52] Khosravi-Darani and Mozafari [50]
Types of SCF			
Carbon dioxide	Low toxicity and reactivity Moderate critical temperature and pressure Good availability Low cost Non-flammable	Low dielectric constant	Hejazi *et al.* [49] Luque de Castro and Jiménez-Carmona [74]
Ammonia	Better solvent strength than carbon dioxide	Difficult to pump Chemically reactive Dangerous for routine use	Luque de Castro and Jiménez-Carmona [74]
Methanol	Excellent solvent	High critical temperature Liquid at ambient temperature, therefore complicates extraction process	Luque de Castro and Jiménez-Carmona [74]

3.5. Cell fragility

Increase in osmotic fragility during the accumulation of PHA are well documented with some microorganisms such as *Azotobacter vinelandii* UWD and recombinant *E. coli* (Table 1) [54–55]. The cell wall strength of these microorganisms could be compromised by modifying the composition of the growth medium. Cell fragility mechanism is not only restricted to Gram negative microorganisms but could be also exploited for Gram positive microorganisms [56]. Page and Cornish [55] demonstrated that the addition of fish peptone to the cultivation medium of *A. vinelandii* UWD led to the formation of large, pleomorphic, osmotically sensitive cells while high molecular weight P(3HB) synthesis was enhanced. About 92% of P(3HB) could be quickly extracted from the fragile cells with 1 N aqueous NH_3 (pH 11.4) at 45°C for 10 min. The same phenomenon was observed with *B. flexus* [56]. The cells grown in the inorganic salts medium suffered from the absence of diaminopimelic acid (DAP) and decreased concentrations of other amino acids. DAP is an important component that formed cross bridge in the peptidoglycan and have a great influence on the structural stability of the cell wall. Up to 86–100% PHA recovery was obtained using hot chloroform or mild alkaline hydrolysis with cells cultivated in inorganic medium while only 32–56% of PHA was able to be extracted from cells grown in organic medium supplemented with peptone or yeast. Simple recovery method with mild extraction conditions could be developed based on cell fragility. However, it is necessary to balance the cell wall softening and cell wall integrity [55] in order to promote microbial growth with higher PHA content (Table 2).

3.6. Flotation

Ibrahim and Steinbüchel [57] investigated the recovery of P(3HB) from a recently isolated bacterium, *Zobellella denitrificans* MW1 (Table 1). Simple extraction with various organic solvents followed by self-flotation of cell debris was tested. The cells were mixed with chloroform at 30°C for 72 h and later subjected to self-flotation of cell debris overnight at room temperature. This method allowed efficient recovery of 85% (w/w) of P(3HB) with purity of 98%. This method should be cost effective as additional steps such as centrifugation and wastage of polymer during recovery could be avoided (Table 2) [57]. Adoption of green solvents together with flotation technique perhaps would add more benefits for the downstream processing of PHA. Previously, selective dissolved-air flotation was also applied to extract mcl-PHA from the cell debris of *P. putida* (Table 1) [58]. The main limitation of dissolved-air flotation is that it requires several consecutive flotation steps (Table 2).

3.7. Aqueous two phase system (ATPS)

Another potential method for the recovery of PHA was recently reported using *B. flexus* [37]. Aqueous two phase system (ATPS) is formed when two polymers at low concentrations (or of one polymer and an inorganic salt) that display incompatibility are mixed such that two immiscible phases coexist (Table 1) [59]. PHA containing *B. flexus* cells were subjected to enzymatic hydrolysis of *Microbispora* sp. cells filtrate and later introduced to polymer-salt ATPS system (Polyethylene glycol [PEG] 8000/phosphate, pH 8.0 and 28°C) reported recovery of high molecular weight PHA ($1{\cdot}10^6$) with 97% purity. In addition, the authors highlighted that protease present in the *Microbispora* sp. cells filtrate was also isolated with several fold purity as a byproduct together with PHA [37]. Several factors, i.e. polymer molecular weight, concentration of polymer and salt, pH, molecular mass, charge etc. have to be considered to choose a good ATPS recovery system. This technique is considered attractive because of short processing time, low material cost, low energy consumption, good resolution, high yield and a relatively high capacity [59–60]. However, ATPS is not yet being used in industrial scale due to problems such as robustness, reproducibility, absence of commercial kits to evaluate ATPS at bench scale as well as poor understanding of the mechanism (Table 2) [60].

3.8. Gamma irradiation

The effect of gamma irradiation on the wet cells of *B. flexus* to assist cell disruption for PHA recovery has also been investigated (Table 1) [61]. PHA recovery of 54% (based on biomass dry weight) was attained with the irradiated cells (10 kGy) subjected to chloroform extraction at room temperature in a short period. However, only 18–20% of PHA (based on biomass dry weight) was recovered from

unirradiated cells exposed to chloroform extraction at 37°C and 150 rpm for 2 h. Gamma irradiation offers many advantages such as promoting optimal disruption of cells at low dosage of irradiation which enable easier recovery of PHA. Besides, retention of solvent solubility due to the low degree of cross-linking was reported [61]. Furthermore, radiation induced cell disruption is independent of any chemicals resulting in relatively contamination free process. The major setbacks of this technique are the length of irradiation time and the initial investment cost that hinders large scale applications (Table 2) [62].

The chemicals and the conditions used for each recovery technique described above are summarized in Table 4. This table clearly shows that chemicals such as organic solvents, alkaline or acidic solutions and surfactants are widely used in most of the PHA recovery methods and have been tested under different working conditions. Even with fragile cells, mild chemicals such as aqueous NH_3 are still required in order to completely rupture the cells. In contrast, enzymatic digestion seems to be more environmentally friendly. However, pure enzymes are costly. In order to address the cost issue, some researchers have used whole microbial cultures as the source of the enzymes. It has been reported that the use of *Microbispora* sp. culture instead of pure enzymes to hydrolyze *S. meliloti* cells and was found to be promising. Some methods like bead mill, high-pressure homogenization and supercritical fluid disruption are also environmentally friendly since no chemicals are involved in the PHA recovery process. The use of gamma irradiation has to be extensively studied to prove its efficiency.

4. Large scale production of PHA

The underlying challenge for the commercialization of PHA is the high production cost which arises predominantly due to expensive purification technologies of PHA. Still, many attempts have been made on the development of simple fermentation strategies as well as modification of recovery techniques in order to scale-up the PHA production.

Table 4. Various chemicals and conditions used in PHA recovery techniques

Recovery method	Strain	Chemical(s) and conditions	Reference
Solvent extraction	*Cupriavidus necator* DSM 545	1,2-propylene carbonate, 130°C for 30 min	Fiorese *et al.* [25]
Digestion with surfactant	Recombinant *Escherichia coli*	Sodium dodecyl sulfate (SDS), 30°C, 1 h	Choi and Lee [54]
Digestion with sodium hypochlorite	*C. necator* Recombinant *E. coli*	Sodium hypochlorite, 30°C, 1 h	Hahn *et al*. [69]
Digestion with surfactant-sodium hypochlorite	*Azotobacter chroococcum* G-3	SDS, 55°C, 15 min followed by sodium hypochlorite, 30°C, 3 min	Dong and Sun [28]
Digestion with surfactant-chelate	*Sinorhizobium meliloti*	Triton X-100 mixed with EDTA, 50°C, 10 min	Lakshman and Shamala [34]
Dispersion of sodium hypochlorite and chloroform	Bacillus cereus SPV	50 ml chloroform : 50 ml sodium hypochlorite, 38°C, 1 h	Valappil *et al.* [31]
Selective dissolution by protons	Cell slurry	0.1 M H_2SO_4, 100°C, 120 min followed by pH adjustment (pH 10) and decolorization using bleaching solution, 2 h, room temperature	Yu [22]
Enzymatic digestion	*S. meliloti*	*Microbispora* sp. culture-chloroform	Lakshman and Shamala [34]
Bead mill	*Alcaligenes latus*	Bead diameter of 512 μm, bead loading of 85%, 2800 rpm	Tamer *et al.* [23]
High pressure homogenization	*Methylobacterium* sp V49	Operating pressure 400 $kg \cdot cm^{-2}$, 5% (w/v) SDS	Ghatnekar *et al.* [48]
Supercritical fluid	*C. necator*	Supercritical CO_2, 100 min, 200 atm, 40°C and 0.2 ml of methanol	Hejazi *et al.* [49]
Cell fragility	*Azotobacter vinelandii* UWD	1 N aqueous NH_3 (pH 11.4), 45°C 10 min	Page and Cornish [55]
Self flotation of cell debris	*Zobellella denitrificans* MW1	Chloroform, 30°C, 72 h, Self flotation of cell debris overnight at room temperature	Ibrahim and Steinbüchel [57]
Aqueous two phase system	*B. flexus*	Polyethylene glycol [PEG] 8000/phosphate, pH 8.0 and 28°C, 30 min	Divyashree *et al.* [37]
Gamma irradiation	*B. flexus*	Radiation doses of 10–40 kGy	Divyashree and Shamala [61]

Chen *et al.* [63] investigated the accumulation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from *Aeromonas hydrophila* 4AK4, which was cultivated in a 20 000 l fermentor using lauric acid as the carbon source under phosphorus limitation. A maximum cell dry weight (CDW) of 50 g/l and PHA content of 50 wt% was achieved by optimizing the carbon source concentrations and the time point for applying phosphorus limitation. The cultivated cells were initially precipitated by adding 1% Na_2HPO_4, 1% $CaCl_2$, and 100 ppm polyacrylamide into the fermentor and filter-pressed to remove water. The cakes of cells were then freeze-dried and ground into powder. PHA extraction was carried out by gently stirring 200–500 kg of dried cells at 60°C for 2 h in 30 000 l extraction tank containing 5000 l of ethyl acetate. The polymer solution was passed through a metal filter and centrifuged to remove cell debris before performing precipitation using 15 000 l of hexane or heptane. Finally, the flocculants of polymer were collected using filter press followed by washing with ethanol before being vacuum-dried. The use of solvents such as ethyl acetate, hexane or heptane were thought to reduce the PHA recovery cost, which accounted to more than 50% of the overall PHA production cost since the solvents could be recovered by distillation. The idea of acetone-water based PHA extraction was proposed to make the recovery process more economically feasible. Recently, large-scale cultivation of *P. putida* GPo1 and recovery of poly(3-hydroxyoctanoate) (PHO) were studied [64]. As high as 53 g/l of CDW with 60 wt% PHA accumulation was obtained by manipulating the inoculum size (1–2%), pH of the medium as well as carbon (octanoic acid) and nitrogen (ammonium octanoate) source concentrations. Continuous separation together with continuous cell release from the separator was used to separate the cells before the freeze drying process. The recovery process involved 25 kg freeze-dried cells in 250 l of acetone which was later concentrated to 75 l. Acetone as an alternative to chlorinated solvents, is an inexpensive, relatively safe and readily available renewable resource, which can be produced as a by-product of microbial fermentation. The acetone recovered through distillation was used several times to lower the extraction costs. The recycling strategy applied in this research [64] was similar to the work described by Chen and co-workers [63]. Precipitation solvents composed of 70% (v/v) methanol and 70% (v/v) ethanol has also been evaluated. This mixture overcomes the problem of 10 volumes of PHA non-solvent consumption for precipitation of the polymer when acetone was used as a solvent. Instead, 1:1 (v/v) ratio of the mixture to PHO concentrate was sufficient to isolate 94% of PHO with 99% purity [64]. Comparison of the two PHA recovery methods reported using different types of solvents suggests the latter to have more profound economical impacts. The best option is to reduce the extensive use of solvents. If acetone-water based recovery method could be developed and extended for extraction of various types of PHA, it will be possible to reduce the extraction cost.

5. Summary and outlook

Microbial PHA is a potential renewable biopolymer with properties closely resembling some common petrochemical plastics. Because of the vast range of structurally different monomers that can be polymerized by microbes, a wide range of material properties can be achieved. Metabolic engineering and high-density cell culture technologies can be exploited for the large-scale production of specified PHA. Based on information reported in the literatures, it is not impossible to grow bacterial cells up to a density of 150 g/l with a PHA content of more than 80 wt% of the cell dry weight. However, the extraction and purification of the PHA granules from the cell biomass is a challenging task especially when one considers the use of environmentally hazardous chemicals as an unacceptable option in the production of eco-friendly materials. This paper has reviewed the various types of methods that have been tested for the extraction and purification of PHA granules from microbial cell biomass. While cost is a major deciding factor in the selection of a suitable method, it is generally expected that the usage of strong chemicals and solvents need to be minimized. The final intended application for the PHA will determine the degree of purity of the PHA granules. For example, in medical applications it is absolutely necessary that the PHA should be free from bacterial endotoxins and other contaminating chemicals and solvents. On the other hand, if the PHA is intended for applications such as mulching

film or garbage bags, a lower degree of purity may be acceptable. Regardless of its final applications, the molecular weights of the recovered PHA should be sufficiently high. This is because the thermal processing of the PHA would result in the reduction of its molecular weights to some extent. Therefore, it is important to have PHA resins with as high a molecular weight as possible. In order to obtain PHA with a high degree of purity, more stringent recovery process is needed. This often results in PHA with lower molecular weights. In addition, the recovery yield will also be lower. Therefore, the challenge in the recovery process is to maintain the original molecular weights while not compromising the degree of purity for various applications. These criteria have to be achieved in an environmentally friendly manner. Finally, and most importantly, the cost of the recovery process should be economically feasible. Together with PHA recovery techniques, fermentation strategies also should be governed. Development of strains that could effectively overproduce PHA from various unprocessed, cheap and renewable carbon sources also plays an important role in lowering the cost of PHA production. Commercialization of PHA could be accomplished by broadening the PHA application areas and exploring more high value added usage such as medical applications.

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