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A Sustainable Approach for the Downstream Processing of Bacterial Polyhydroxyalkanoates: State-of-the-art and latest developments

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1 # *A Sustainable Approach for the Downstream Processing of Bacterial*
2 # *Polyhydroxyalkanoates: State-of-the-art and latest developments*

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Abstract:

3 Bioplastics have emerged as a platform to reduce our dependence on fossil fuels.
4 Polyhydroxyalkanoates (PHAs) are a family of biodegradable polyesters with large potential in
5 consumer goods and medical applications. These polymers accumulate in prokaryotic microbes
6 and their recovery is a challenging, often under explored, operation. In the past, oil-derived
7 solvents and chemicals have been widely used as extracting agents, compromising the
8 "environmentally-friendly" claim of bioplastics. Furthermore, the large amount of chemicals
9 and solvents required at the industrial level would negatively impact the economics of the
10 process. The present review presents the latest advances in the field of downstream operations
11 for PHA recovery emphasizing those green technologies with scaling-up feasibility. As for the
12 upstream and fermentation stages, the extraction process needs to be carefully optimized to
13 accomplish a competitive production of PHAs.
14

Keywords: bioplastics, PHAs, downstream operations, green solvents, digestion, recovery, sustainability

List of symbols:

ATPE	Aqueous two-phase extraction	**MIBK**	Methyl isobutyl ketone
CAGR	Compound annual growth rate	**MMC**	Mixed microbial cultures
CP	Cloud point	**MTBE**	Methyl *tert*-butyl ether
DMC	Dimethyl carbonate	M_w	Molecular weight
DSP	Downstream processing	**NPCM**	Non-polymer cellular matter
EDTA	Ethylenediaminetetraacetic acid	**PEG**	Polyethylene glycol
EOPO	Ethylene oxide propylene oxide	**PHA**	Polyhydroxyalkanoate
HPH	High pressure homogenization	**P(3HB)**	Poly(3-hydroxybutyrate)
ICI	Imperial Chemical Industries	**P(3HHx)**	Poly(3-hydroxyhexanoate)
LAS	Linear alkylbenzene sulphonate	**P(3HO)**	Poly(3-hydroxyoctanoate)
LCA	Life cycle assessment	**P(3HV)**	Poly(3-hydroxyvalerate)
LDPE	Low density polyethylene	**PP**	Polypropylene
LPS	Lipopolysaccharides	**scl**	Short-chain length
mcl	Medium-chain length	**sCO2**	Supercritical carbon dioxide
MEK	Methyl ethyl ketone	**SDS**	Sodium dodecyl sulphate

15 ## 1 INTRODUCTION

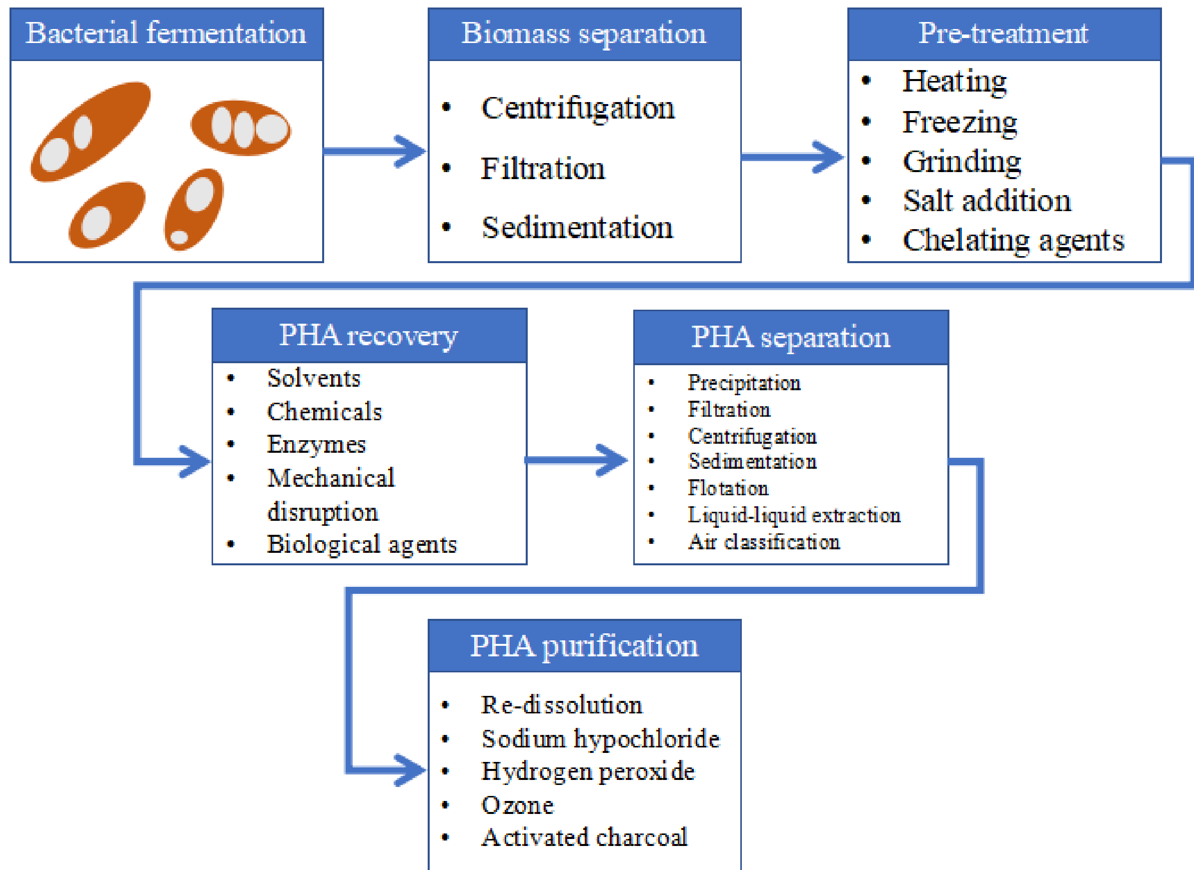
16 Oil-derived plastics have grown at a faster rate than any other bulk material for several decades.
17 Biobased polymers could serve to offset, to a certain extent, the non-renewable feedstock used
18 in the plastic industry. Moreover, biodegradability is seen as a solution to the major plastic
19 disposal problem. However, the process development of biobased biodegradable polymers is
20 certainly at a very early stage compared to the petrol-based plastic manufacturing industry.
21 Over the last years, intensive research has targeted the optimization of bioplastic production.
22 The primary objective is that large-scale operation can compete with that of traditional plastics
23 while reducing the environmental impact [1].

24 Microbially produced plastics are promising candidates for a biobased generation of
25 biopolymers. Great efforts have focused in upstream operations for example, in the selection
26 and engineering of prokaryotic and eukaryotic strains, as well as in the utilisation of cheap

27 substrates for their production [2,3]. Nevertheless, developments in the purification and
28 recovery of bioplastics has been rather slow. Moving towards industrialization, it is clear that
29 the production of biopolymers needs to go hand in hand with eco-friendly downstream
30 operations [4].
31 The global polyhydroxyalkanoates (PHAs) market size is projected to reach 23,734.65 metric
32 tons by 2021, at a compound annual growth rate (CAGR) of 6.27 %, between 2016 and 2021
33 [5]. These biopolymers exhibit a wide range of properties derived from the structural variation
34 of their backbone/side chains and have demonstrated an outstanding biocompatibility [6]. PHAs
35 can be classified, depending on the number of carbon atoms in the monomer unit, into short-
36 chain length (scl) PHAs (3 to 5 carbon atoms) and medium chain length (mcl) PHAs (from 6
37 to 14). Scl-PHAs can be used to produce rigid plastics with properties comparable to those of
38 polypropylene (PP) whereas mcl-PHAs imitate more flexible materials like low density
39 polyethylene (LDPE). PHA degradation has been reported to occur in soil, fresh and salt water
40 and in the human body, although the degradation time needs to be carefully evaluated before
41 selecting a disposal route [7].
42 Polyhydroxyalkanoates (PHAs) are energy and carbon reservoirs [8] polymerized and stored
43 within the host cells. This condition makes their recovery more challenging than the separation
44 of some other (extracellular) fermentation products. In the past, chlorinated and other oil-
45 derived solvents, harsh chemicals and energy intensive practices have been used to demonstrate
46 the feasibility of producing PHAs from a microbial culture [9]. We have now reached a point
47 where downstream processing (DSP) for the recovery of bioplastics cannot jeopardize the
48 environmental impact of a well-established bioprocesses.
49 The focus of this review is to provide the reader with an overview of the more relevant options
50 for scl and mcl-PHA recovery, from pure and mixed cultures, and stress the environmental
51 impact associated with each one of them. Although numerous reviews on PHAs are available,
52 the emphasis of this work is to critically discuss the most recent DSP methods and highlight
53 those with scalability potential and low ecological burden.

54 ## 1.1 Production of PHAs

55 PHAs are synthesized by different organisms, including archaea, bacteria, yeast, algae, plants
56 and their recombinant forms. Bacterial cultivation can be carried out in pure or mixed culture.
57 PHA generation can be coupled or decoupled to cellular growth, and frequently the limitation
58 of an essential nutrient (nitrogen, phosphorus, oxygen, etc.) and an excess of carbon is exploited
59 to trigger polymer accumulation. PHA is water insoluble and accumulates as chains surrounded
60 by diverse proteins in cytoplasmatic granules. In some highly optimised microbial producers,
61 PHAs can account for more than 90% of the bacterial dry cell weight in the form of multiple
62 granules [10].
63 The process of producing PHAs includes a series of steps, within which downstream operations
64 can account for half of the production costs [11]. The first stage after fermentation is to separate
65 the biomass (cells containing PHAs) from the broth. Centrifugation, filtration, sedimentation
66 are the most common methods to achieve this. Biomass can be pre-treated to increase the
67 permeability of the bacterial cells by heating, freezing, adding salts, grinding in liquid nitrogen
68 and using hot compressed water. Biomass, pre-treated or not, is then subjected to an extraction
69 process in which there is either a solubilization of cellular material surrounding the PHA, or
70 solubilization of the polymer itself. PHA is subsequently separated from the disrupted cellular
71 matter and purified according to the final requirements of the final product as shown in Figure
72 1.



73 **Figure 1: Conventional stages involved in the recovery of PHA from bacterial fermentation and most common**
74 **methods for each stage.**

75 # 2 RECOVERY METHODS

76 ## 2.1 Solvents

77 Solvent extraction is to date the most widely spread practice for the recovery of PHAs. There
78 are certain solvents that can alter the permeability of the cell membrane and selectively dissolve
79 the polymer stored inside. In some cases, a pre-treatment step is used to increase the solvent
80 accessibility to the polymer. After being dissolved, PHA is recovered with a precipitating agent
81 e.g. ethanol or methanol, at low temperatures.

82 ### *2.1.1 Halogenated solvents*

83 Halogenated solvents such as chloroform, methylene chloride, 1,2-dichloroethane, 1,1,2-
84 trichloroethane and 1,1,2,2-tetrachloroethane have been tested to recover PHAs [12,13].
85 Lemoigne, who first discovered P(3HB) in 1926, was also the first to use chloroform for
86 extraction and it is still the reference technique for comparison purposes. The Soxhlet set-up

87 allows the recirculation of hot solvents, to maximize solubility and reduce the large volumes of
88 solvents used for the extraction. It provides good recovery yields and high purity of the
89 extracted polymer [14]. The chloroform method allows the extraction of polymer with low
90 endotoxin content, an important requirement when the polymer is targeted for medical
91 applications.

92 The natural morphology of the PHA granules can be affected by the use of halogenated
93 solvents, which prevents the use of PHA for certain applications, for example in the production
94 of strong fibres [15]. Additionally, the large amounts of solvents (20 parts of solvent per one
95 part of polymer) make this approach an expensive option for application at industrial scale.
96 Furthermore, chlorinated solvents are harmful compounds, suspected to induce cancer, that
97 cause long-term adverse effects in aquatic environments and believed to contribute to ozone
98 layer depletion. Their use is banned in consumer products in several countries, and thus, they
99 should be replaced with safer alternatives.

100 ***2.1.2 Halogen-free solvents***

101 In view of the negative effects of halogenated solvents, a series of patents from companies such
102 as Agroferm, Procter & Gamble, Monsanto, Metabolix and Kaneka describe the use of
103 alternatives to chlorinated solvents [16–21]. Table 1 lists the main halogen-free solvents used
104 in the last decade.

105 Kurdikar and co-workers [18] investigated mixtures of non-halogenated PHA-solvents:
106 alcohols, esters, amides, cyclic and acyclic ketones, which can be used for PHA recovery and
107 Narasimhan et al. [22] studied the influence of high temperature when using these types of
108 solvents. Acetone extraction at high temperature and pressure was studied by Koller and co-
109 workers to recover scl-PHA. As advantages, the method enabled the reutilization of the solvent
110 and reduced the extraction time from 12 h to 20 minutes. Performance compared to that of
111 cholorofom recovery [23].

112

113

114 One of the advantages of the chloroform-based method was the ability to reduce the endotoxin
115 content in the final polymer; Furrer et al. achieved an endotoxin level between 10 and 15 U/g
116 P(3HO) by using n-hexane followed by 2-propanol [24].
117 According to Chemat, “*Green Extraction is based on the discovery and design of extraction*
118 *processes which will reduce energy consumption, will allow the use of alternative solvents and*
119 *renewable natural products, and ensure a safe and high quality extract/product”* [25].
120 Halogen-free solvents are in general less harmful than chlorinated solvents but are not
121 completely sustainable. Many of them are oil derived and require special treatment for disposal.
122 To assess the risk of a solvent, GSK have created a solvent selection guide that considers health,
123 reactivity and stability, environmental impact, life cycle score, legislation flag, environmental
124 health and safety flag, boiling and melting points and waste disposal [26].
125 To date dimethyl carbonate (DMC) and biobased solvents are seen as the most eco-friendly
126 alternatives for extraction [27]. DMC is completely biodegradable, whereas ethyl acetate, ethyl
127 lactate, butyl lactate and isopropanol can be produced through biochemical conversion of
128 biomass. Similarly, ethanol produced through biomass fermentation would have a lower impact
129 on the overall non-renewable carbon footprint of the process. A good example of an integrated
130 biorefinery in Brazil uses waste streams from ethanol distillation as solvents to recover PHAs
131 [28].
132 Fei *et al.* proposed an ‘environmentally friendly’ process for P(3HB) recovery, from *C. necator*
133 cells using a solvent mixture of acetone, ethanol and propylene carbonate in the same volume
134 proportions, starting with non-defatted wet biomass. When hexane was selected for polymer
135 precipitation, improved polymer yield and purity were obtained (92 and 93% respectively).
136 Physical properties did not differ from chloroform-extracted P(3HB) [29].
137 **Table 1: Studies on halogen-free solvents for PHA recovery.**

Hydrocarbon	Hexane	P(3HO), P(3HB)	[30–32]
Alcohol	Methanol	P(3HB)	[31,33]
	Ethanol	PHA	[33,34]
	Propanol	PHA	[31,33]
	C1 to C6 alcohol	PHA	[35]
Ketone	Acetone	scl-PHA, P(3HO)	[23,36,37]
	Methyl isobutyl ketone (MIBK)	P(3HB-*co*-3HHx)	[20,38]
	Methyl ethyl ketone (MEK)	P(3HB-*co*-3HHx), P(3HO-*co*-3HV)	[38,39]

	Cyclo-hexanone	P(3HB)	[20,40,41]
Lactone	γ-butyrolactone	P(3HB)	[41]
Acid	Acetic acid	P(3HB), PHA	[33,42]
	Acetic acid anhydride	PHA	[43]
Carbonate ester	Dimethyl carbonate (DMC)	PHA	[44]
	Ethylene carbonate	P(3HB)	[16,33]
	1,2-propylene carbonate	P(3HB), PHA block copolymers	[16,45,46]
Ester	Methyl lactate	PHA	[47]
	Ethyl lactate	P(3HB-*co*-3HHx), PHA	[31,38,47]
	Ethyl acetate	P(3HB-*co*-3HHx)	[48]
	Butyl acetate	P(3HB-*co*-3HHx)	[20,31,38]
Organosulfur	Dimethyl sulfoxide	P(3HB)	[33]
Amide	Dimethyl formamide	P(3HB)	[33]
Cyclic ether	Tetrahydrofuran	P(3HB)	[17,49,50]
Lactam	n-methyl-pyrrolidone	P(3HB)	[51]
Ether	Diethylether	mcl-PHA	[52]
	Methyl *tert*-buthyl ether (MTBE)	mcl-PHA	[53]
	Anisole	P(3HB)	[40]
	Phenitole	P(3HB)	[40]

138 In the search for "green solvents", ionic liquids have also been investigated to substitute the
139 large amount of volatile solvents and undesirable chemicals used in extraction processes. These
140 compounds have low vapour pressure, therefore, produce no hazardous gases. Ionic liquids are
141 salts with a melting temperature of 100°C or less which can dissolve substances that are
142 insoluble in water [54]. The patent, owned by Procter & Gamble [54], describes how PHAs can
143 be extracted in a batch or continuous system (stripping) by simple contact with an ionic liquid
144 in the absence of water. Polymer is then isolated from the ionic liquid with a recovery solvent,
145 such as an alcohol, followed by conventional separation methods (sedimentation,
146 crystallization, centrifugation, decantation, filtration or combination of those). A particular case
147 was reported by Kobayashi et al. who investigated the power of ionic liquids to dissolve NPCM
148 from cyanobacteria. They reported that 1-ethyl-3-methylimidazolium methylphosphonate was
149 able to dissolve cyanobacteria components but not P(3HB) [55].

150 2.2 Digestion methods

151 In ideal conditions the NPCM content should be less than the polymer, therefore, dissolving
152 the former should be, in principle, relatively less challenging.

153 *2.2.1 Chemicals*

154 <u>Sodium hypochlorite</u>

155 Sodium hypochlorite is a strong oxidizing chemical able to dissolve proteins, lipids,
156 carbohydrates and nucleic acids that constitute the non-PHA matter. Although a very pure
157 polymer (over 95%) can be recovered using sodium hypochlorite, the reduction of the
158 molecular weight of the polymer after treatment with this harsh chemical has led to serious
159 concerns [56].
160 The relationship between polymer degradation, volume of sodium hypochlorite and
161 temperature was studied in *Cupriavidus taiwanensis* 184. Increasing the volume of sodium
162 hypochlorite up to 30 mL per g of dry cells, at constant temperature (50°C), resulted in 80%
163 reduction in molecular weight. The effect of temperature, at constant volume of the oxidizing
164 agent (2 mL) was less deleterious but still significant: almost a 30% decrease in molecular
165 weight was observed when temperature was increased from 40 to 100°C [57].
166 In order to tackle the M_w decrease, the combined effect of chloroform and sodium hypochlorite
167 was implemented by Hahn et al. [58]. In the so-called dispersion method, lyophilised biomass
168 was incubated with sodium hypochlorite and chloroform for 2 hours at 30□ in an orbital shaker
169 at 140 rpm (5 mL of 80% sodium hypochlorite solution in distilled water and 22.5 mL of
170 chloroform were added per 0.3 g biomass). The hydrophobic P(3HB) dissolved into chloroform
171 as soon as cells lysed, avoiding polymer destruction by hypochlorite. After incubation, the
172 slurry was centrifuged which resulted in phase separation and three layers were formed. The
173 top two layers were sodium hypochlorite and cell debris. The bottom layer, containing
174 chloroform and dissolved polymer was collected, filtered and concentrated. Polymer was
175 precipitated using ice-cold methanol in a 1:10 ratio under continuous stirring.
176 Successful studies have been carried out using just sodium hypochlorite on a high-rate
177 continuous process and at a large scale extraction process [59,60]. In the first case, yield of
178 polymer recovery (around 100% w/w) and purity (more than 90% of PHA content in the
179 residual solids, on a weight basis) was achieved even though it was a mixed culture system.
180 Also in MMC, Samorì et al. [44] employed a combination of a green solvent, DMC, and the
181 use of sodium hypochlorite in a pretreatment step.
182 <u>Sodium and potassium hydroxide</u>

183 The saponification reaction between sodium hydroxide and the lipid layer in the bacterial cell
184 wall destabilises the membrane and increases its permeability [61]. Sodium hydroxide or
185 potassium hydroxide are mild digestion agents which can overcome some of the environmental
186 concerns related to the use of harsh chemicals for PHA recovery.

187 Mohammadi et al. investigated the digestion conditions with a Gram-negative PHA-
188 accumulating bacteria, *Comamonas* sp. EB173. They found that a sodium hydroxide
189 concentration of 0.05 M and a digestion time of 1 h at 4°C gave the best results and achieved
190 an 88.6% purity and a 96.8% recovery yield. Purification of the polymer was simply performed
191 with ethanol and water. The same parameters were evaluated with recombinant *C. necator* cells.
192 Although the length of the treatment was extended to 4 h in order to optimize the results, PHA
193 was effectively recovered even from cells with low PHA content [62,63].

194 A simple procedure was adopted by Anis et al. [64] to recover P(3HB-*co*-3HHx) from
195 recombinant *C. necator* cells. Best results were found when freeze dried biomass, in
196 concentration ranging from 10 to 30 g/L, were incubated in 0.1 M sodium hydroxide from 1 to
197 3 h at 30°C and the polymer polished using 20% (v/v) of ethanol. Under such conditions, the
198 recovered copolymer P(3HB-*co*-3HHx) could reach 80 to 90% (w/w) of purity and recovery
199 yield.

200 Two digestive solutions, sodium hydroxide (1 M) and sodium hypochlorite (5% chlorine) were
201 evaluated in an extraction reactor fed with biomass with a high PHA content. The fermentation
202 system consisted of a high-rate continuous process with an enrichment step of the MMC.
203 Sodium hydroxide was less effective than the sodium hypochlorite in the overall PHA recovery
204 both in the 3 h and 24 h treatment. The action of sodium hypochlorite (5% chlorine) resulted
205 in a total recovery of the polymer with more than 90% (w/w) of PHA content in the residual
206 solids. The relatively lower performance of sodium hydroxide can be attributed to the additional
207 difficulty of cell disruption in mixed cultures [59].

208 Recently, Irdahayu et al. [65] proposed a recovery strategy based on the synergistic effect of
209 sodium hydroxide and Lysol, a commercial detergent, for non pre-treated biomass. The
210 methodology was applied to batches with varying 4HB monomer content. Beside the good

211 recovery, 90% at the largest extraction volume (100 L), the polymer exhibited high purity,
212 retained its original properties and could be suitable for biomedical applications.

213 Acids

214 Acids can also be used to disrupt the non-PHA cellular material (NPCM) and liberate the
215 intracellular PHA. With the view to identifying a cost-effective recovery system and assess the
216 environmental impact of the downstream operations, López-Abelairas et al. [66] compared
217 sulphuric acid with three other alkaline solutions (sodium hydroxide, sodium hypochlorite and
218 sodium hypochlorite combined with dichloromethane). The lowest costs were those associated
219 with the use of sodium hydroxide and sulphuric acid (1.02 and 1.11 €/kg respectively). In
220 addition, the CO_2 emissions of these two chemicals were only 18% of the emissions produced
221 by sodium hypochlorite. Sulphuric acid was able to extract the purest polymer without polymer
222 degradation thus, it was selected as the most appropriate choice.

223 Yu and Chen [67] developed a promising method based on the selective dissolution of NPCM
224 in aqueous acidic solution and crystallization of biopolymers. P(3HB) from *Ralstonia eutropha*
225 cells was extracted with a 97.9% (w/w) purity and 98.7% (w/w) recovery. Even a copolymer,
226 P(3HB-*co*-3HV) and terpolymer, P(3HB-*co*-3HV-*co*-4HV) were successfully recovered with
227 very high values of purity and yield: 98.5% (w/w), 95.4% (w/w) respectively for the former
228 and 96.4% and 94.8% (w/w) for the latter. Average molecular weight was described as a
229 function of processing conditions. The processing parameters need to be carefully controlled in
230 order to avoid a major reduction in the original value.

2.2.2 Surfactants

231 Surfactants enter the lipid membrane increasing the volume of the cell envelop until it bursts.
232 Micelles of surfactants and membrane phospholipids are then formed and PHA granules
233 released. Surfactants can also solubilize proteins and other molecules from the NPCM.

234 Anionic sodium dodecyl sulphate (SDS) is the most widely used surfactant in PHA recovery.
235 This detergent is known for its ability to recover genetic material. SDS can be directly added
236 in high cell density cultures of *R. eutroph*a cells and it has demonstrated a good recovery

regardless of the inclusion or not of a pretreatment option [68]. Other surfactants, such as the synthetic palmitoyl carnitine, naturally synthesized in mammalian cells as part of the fatty acid metabolism, were used with *R. eutropha* and *Alcaligenes latus* cells and exhibited a stronger lytic activity in the latter case [69].

Very high purities cannot be achieved with surfactants only. Therefore, a combination with some other chemical or enzymatic treatment is the most usual practice. The influence of sodium hydroxide, sodium hypochlorite and chelating agents, together with SDS, have been demonstrated and purities up to 99% have been reached [70]. Surfactant concentration, in aqueous solution, needs to be kept below 5% (w/w) in order to avoid a disposal problem or increased cost. SDS concentrations in the range of 0.025 to 0.2% (w/w) have been found to be sufficient for the process [71]. Biobased surfactants or biodegradable detergents, such as linear alkylbenzene sulfonic acid (LAS-99), could be sustainable alternatives to reduce the disposal efforts related to traditional surfactants [72].

2.2.3 Enzymes

An enzymatic process can also be used to lyse and digest major parts of NPCM. Cocktails of proteases, nucleases, phospholipases, lysozymes and other enzymes, in combination with surfactants and chelating agents (and heat treatment to accelerate degradation), have been known for a long time as mechanisms to recover PHAs [73–75]. For example, the added effects of alcalase (digest denatured proteins), SDS (solubilization) and EDTA (assist solubilization by complexing the divalent cations and thus destabilizing the membrane fragments) were selected by de Koning and Witholt [76] to extract mcl-PHAs.

The Zeneca process developed by Imperial Chemical Industries (ICI) started with an intense heat treatment of the PHA-rich biomass. Enzymatic hydrolysis with pepsin, trypsin and papain was followed by a surfactant dissolution of the residual cellular matter. Finally, a decolorization step with hydrogen peroxide of the isolated polymer was performed [74]. Enzymes from *Cytophaga* species demonstrated their potential in lysing *R. eutropha* cells completely at 37.5°C, a pH of 7.3, in 60 minutes of incubation, with no mechanical treatment involved [77].

264 Kapritchkoff et al. [78] screened different enzymes to recover P(3HB) from *R. eutropha* cells,
265 discovering that trypsin, bromelain and lysozyme were found to be the most promising.
266 A concentration of 2% (w/w) bromelain, at 50°C and pH 9 gave a polymer with 88.8 % purity.
267 Using pancreatin instead, the purity was increased to 90%, the costs associated with enzymes
268 were reduced three times and the polymer did not undergo any apparent degradation.
269 Yasotha et al. [79] investigated the recovery of mcl-PHA from *P. putida* cells. They found that
270 the contribution of alcalase was the most important from a mixture of alcalase, lysozyme,
271 EDTA and SDS. Cross-ultrafiltration was used to separate the granules and purification of the
272 polymer was carried out by continuous defiltration.
273 Neves and Müller [80], evaluated several commercial enzymes among proteases and glycosides
274 for their ability to recover P(3HB) and the co-polymer P(3HB-*co*-3HV) from *C. necator* cells.
275 After optimization, 93.2% recovery and 94% purity were obtained with an enzyme solution at
276 0.02% (w/w) of Celumax®, a glycosidase, after 1 hour incubation at pH 4 and 60°C.
277 Lakshman and Shamala [81] cultivated *Microbispora* species on the thermally inactivated
278 fermented broth of *Sinorhizobium meliloti* for 24, 48 and 72 h. PHA was isolated using
279 chloroform or a mixture of a non-ionic surfactant (Triton X-100) and EDTA with a polymer
280 recovery of 98, 82 and 14% respectively for the different incubation times. Alternatively, they
281 simply used the supernatant of *Microbispora* fermentation broth at 72h for hydrolysing *S.*
282 *meliloti* cells and obtained 94% yield and 92% purity.
283 Divyashree and colleagues [82] also used *Microbispora* culture filtrate, containing a protease
284 activity of 3 U/mL, on a *Bacillus flexus* culture. The enzymatic hydrolysis proved to be better
285 than sonication in lysing the cells but less efficient than a sodium hypochlorite digestion.
286 Recently, Israni et al. [83] used the lytic activity of *Streptomyces albus* on *B. megaterium* cells.
287 As in the case described above, two approaches were followed: co-inoculation of *S. albus* with
288 PHA- producer cells and utilization of the lytic culture filtrate for polymer extraction. The
289 enzyme-based extraction led to a 1.74-fold increase in the PHA yield as compared to co-
290 inoculation, attributable to the utilization of the released polymer by the growing *S. albus*. The

lytic activity of *S. albus* was demonstrated in a wide spectrum of Gram positive and negative bacteria, including *B. subtilis* and *P. aeruginosa P6*.

Kachrimanidou and co-workers [84] used the crude enzymes produced by *Aspergillus oryzae* in solid state fermentation to recover P(3HB-*co*-3HV) from *C. necator*. Almost 90% of cells were lysed under optimized temperature and pH conditions. Enzymatic lysis of bacterial cells was also carried out at the optimum temperature and uncontrolled pH value leading to a recovery yield and purity of 98% and 96.7%, respectively. Besides, the author evaluated the recycle of cell lysate for further P(3HB) production.

2.2.4 Biological agents

An innovative approach was introduced when entire organisms were used in the process of recovering intracellular products. These organisms digested the cellular matter from PHA accumulating bacteria while leaving PHA intact [85]. The biopolymer was then recovered from the faecal pellets and simply washed. The nutritional value of *C. necator* cells had been previously reported when this type of bacteria was used as single cell protein for rats [86]. The drawback of a long recovery time can be overcome if the method is integrated into other processes such as insect farming.

2.3 Supercritical fluids

Substances above their critical pressure and critical temperature exhibit an intriguing intermediate behaviour. They have the diffusivity properties of a gas and the solvation power of a liquid. For this reason, supercritical fluids can diffuse through solids and dissolve materials [87].

Supercritical carbon dioxide or sCO_2 is the most common type of supercritical fluid used in biotechnology. It is chemically inert, non-toxic, non- flammable and is readily available at high purity and low cost. Additionally, its temperature and pressure values (31°C and 74 bar) allow working in mild conditions and the residual solvent can be simply vaporized by reducing the pressure, leaving no harmful products for disposal. Based on all these features, it is considered a green solvent [88].

318 Supercritical fluid extraction (SFE) has been applied in industry for the recovery of high-value
319 products such as essential oils and flavours and in the production of decaffeinated coffee and
320 cholesterol-free butter [89]. Since it is a batch process, it rarely applies to inexpensive
321 commodity products but is considered a promising tool for pharmaceuticals and biomedical
322 materials intended for tissue engineering and drug delivery [90].
323 Hampson and Ashby [91] tried to implement SFE to recover mcl-PHAs synthesized by
324 *Pseudomonas resinovorans.* They used lyophilized cells and a fluid flow of 1.5 L/min.
325 Extraction conditions ranged from 40 to 100°C and 2000 to 9000 psi. sCO_2 was used for the
326 extraction of the lipid materials which accounted from 2 to 11% of the non-PHA cellular matter.
327 The increase in the flow rate shortened the extraction time to less than 3 h. A chloroform
328 extraction was still required to recover the polymer, although the SFE step reduced the amount
329 of solvent needed significantly.
330 Confirming the aforementioned results, Williams et al. [92,93] demonstrated that pure
331 supercritical CO_2 is able to extract lipids and other hydrophobic contaminants whereas mixtures
332 with modifiers (conventional solvents) can be later used to extract pure PHA with a good
333 recovery yield. They found that PHA can be soluble at 9% in those mixtures. In this context,
334 Metabolix developed a single step process to recover 100% pure P(3HO) with 25 to 150 times
335 less endotoxin than the one obtained by solvent extraction and recrystallization [94].
336 A few years later, Khosravi-Darani and colleagues [95,96] studied the solubility of P(3HB) in
337 sCO_2 and tested the combination of this technique in a pre-treatment. Interestingly this work
338 found that the cell stage influenced the disruption process. Also, both wet and freeze-dried cells
339 were used but higher purity was found with the latter. Sodium hydroxide was found to be more
340 effective than sodium chloride and resulted in the achievement of a complete disruption after
341 two pressure release events. Hejazi et al. [97] used the Taguchiapo's statistical approach to find
342 the optimum conditions for disruption of *R. eutropha* and P(3HB) recovery using SFE only. An
343 89% recovery was attained using sCO_2 and methanol as modifier for 100 min at 200 atm and
344 40°C.

345 In a more recent review, Koller et al. [4] supported the claim that sCO_2 is good for degreasing
346 PHA rich biomass based on the outcomes of the 5th framework program European project
347 WHEYPOL. These results are in contradiction with the claim that supercritical fluids can be,
348 on its own, a suitable method for PHA recovery. A comparison of the main features of
349 supercritical fluids compared to other solvent and digestion methods is shown in Table 2:

350 **Table 2: Advantages and disadvantages of the extraction and digestion methods for PHA recovery.**

METHOD	STRENGTHS	WEAKNESSES
Solvent extraction	High yield (>90%) High purity (>99%)	Toxicity for human health and environment Some of them are derived from oil
Green solvents	Biobase and/or biodegradable Good performance	Relatively low toxicity Costs in large scale
Chemical digestion	Lower toxicity for human health Low capital investement	Can affect polymer quality Costly waste water treatment/difficult to reuse
Supercritical fluids	Non toxic for human health Environmentally friendly	Not widely availale Recovery mechanism under research
Biological recovery	No chemicals involved Valorization of NPCM	Slow process Low purity

351 ## 2.4 Mechanical disruption

352 ### *2.4.1 Bead mill*

353 Cell disruption by bead milling for the recovery of intracellular products is a common practice
354 in the isolation of DNA, enzymes and recombinant proteins. Complete destruction of the
355 cellular wall, in a non-specific way, is achieved using solid-shear forces generated by the
356 disruption agents, such as glass beads, rotating along with the cell suspension in a chamber
357 [98]. The heat generated in the process needs to be dissipated with a cooling liquid flowing
358 around the grinding chamber.

359 Tamer et al. [99] were the first to study the disruption of *A. latus*. The intracellular release of
360 protein served as an indicator of P(3HB) release. They used heat shock at 80°C to achieve a
361 complete disruption of the cell on 8 passes through the mill. Bead milling was found to be
362 effective independently of biomass concentration and could be used even at low biomass

363 concentrations. The diameter of the beads did not affect the outcome but the beads loading had
364 a strong effect on the results.
365 Disruption processes generally follow first order kinetics and the results that can easily be
366 predicted and scaled up [99]. This fact, together with the relatively low energy consumption
367 and readily available equipment, greatly favours bead milling over other DSP methods.
368 Gutt et al. [100] compared different DSP methods with the same amount of starting *C. necator*
369 biomass and used design of experiments and ANOVA analysis to improve the performance of
370 the better methods found. Mechanical disruption by bead milling coupled with SDS treatment
371 was found to be the best technique and allowed a 100% recovery with 94% polymer purity
372 within two hours.

373 ***2.4.2 High pressure homogenization***

374 A high pressure homogenizer satisfies most of the criteria to be applied at large-scale in DSP
375 [101]. In this piece of equipment, the fermented broth flows at high pressure through a narrow
376 gap where is subjected to very high shear forces that cause cell disruption. Increasing the
377 number of passes in the homogeniser enhances product purity as more of the cellular material
378 is solubilized.
379 When using used high pressure homogenization (HPH) to dirupt *A. latus* cells, Tamer et al.
380 [99] found that the performance of the equipment depended on the biomass concentration. A
381 low efficiency was achieved with low cell density broths. Homogenization of cell
382 concentrations exceeding 66 g/L was neither effective due to process interruption caused by
383 frequent blockages. Furthermore, micronization of P(3HB) to levels where it could not be
384 precipitated was more frequent in the high pressure homogenizer than in the bead mill, resulting
385 in higher losses of the polymer with an increase in the number of passes.
386 Ghatnekar et al. [102] used HPH in combination with 5% (w/v) volume of SDS to attain a 98%
387 yield and 95% P(3HB) purity after two cycles with *Methylobacterium* cells. A cell
388 disintegration of more than 99.99% after a pre-treatment with a strong alkaline solution and 1%
389 SDS solutions followed by HPH has also been reported [4]. Even mechanical digestion with

390 no prior treatment has yielded more than 90% disintegration which highlighted the promising
391 performance of these methods for large scale operation.
392 The amount of the DNA released when cell lysis can represent a challenge for the recovery
393 steps that follow biomass homogenization. Heat treatment, addition of hypochlorite or
394 commercial nucleases are the most common ways of reducing the viscosity and, thereby, easing
395 DSP. To cut down costs, nucleases encoding genes from *Staphylococcus aureus* were inserted
396 in PHA producing strains such as *P. putida* and *C. necator*. In both cases, the lysate viscosity
397 was successfully was reduced without compromising the PHA production [103,104].

398 ### *2.4.3 Ultrasonication*

399 This method uses the power of acoustic waves to break down the cells. High frequency sounds,
400 produced by ultrasonic vibrators, are converted into mechanical oscillation by a transducer
401 through a titanium probe immersed into the cell suspension. Bacterial and fungal cells can be
402 disrupted by ultrasonic means.
403 Ultrasonication has been frequently used as pre-treatment in PHA recovery for different types
404 of cells including *C. taiwanensis* and *Buskholderia* in small scale, although certain species
405 might be more susceptible than others [4]. Penloglou [105] used ultrasonication in combination
406 with a chemical method for the production of a P(3HB) with tailor-made molecular properties.
407 Ishak et al. [106] developed an ultrasound assisted process in which a frequency of 37 kHz
408 facilitated the extraction in a solvent mixture of (acetone)/marginal non-solvent (heptane).
409 Samorì et al. [44] chose glass beads of 0.5 mm diameter and ultrasonication as pre-treatment
410 methods for the non-halogenated solvent extraction process.
411 A process for PHA recovery based on mechanical disruption was patented by Tianan Biological
412 Material Co. Bead milling and ultrasonic processes were used for breaking the cells in the
413 fermentation broth. The pH was adjusted to be alkaline before or after surfactant and coagulant
414 agents were added. The final product was separated after one hour by centrifuge, filter press or
415 vacuum suction filtration. It is claimed by the author that the invention process has low cost,

416 high extraction yield and no pollution. These attributes make it an easily scalable method (Chen
417 2003).

418 A different mechanical process, gamma irradiation, has been explored as cell lysis treatment
419 on *B. flexus* by Divyashree & Shamala [108]. Gamma irradiation of 5 to 40 kGy on wet
420 biomass, obtained after centrifugation of the fermentation broth, resulted in cell damage and
421 favoured the PHA extractability. Irradiated biomass was then subject to chloroform extraction.
422 Although little researched, this method seems to be effective for cell disruption and
423 improvement of polymer properties such as molecular weight and tensile strength increase. A
424 comparison among the mechanical methods presented can be found in Table 3.

425 **Table 3: Advantages and disadvantages of the mechanical methods for PHA recovery.**

METHOD	STRENGTHS	WEAKNESSES
Bed mill	Efficient at low cell concentrations Easily scalable	Several number of passes required Pre-treatment stage involved
HPH	Scale-up potential High yield without pre-treatmetnt	Depends on biomass concentration Micronization of PHA
Ultrasonication	Low cost No pollution	Used in combination with other methods
Gamma irradiation	Can improve polymer properties	Little researched Used in combination with solvents

426 **2.5 Cell fragility**

427 Pre-treatment methods (thermal, pH, osmotic pressure) and the action of solvents, chemicals
428 and/or enzymes are normally applied to increase the vulnerability of the cell wall and liberate
429 the PHA granules. Nonetheless, there are other factors that can indirectly influence the cell wall
430 fragility. For example, Schumann & Müller [51] reported that microorganisms with high PHA
431 content (60 to 80% of dried cell matter) are more fragile and can be easily broken in a few steps.
432 Besides, less chemicals agents, enzymes or chelates are required and there is less risk of
433 polymer damage.

434 Page and Cornish [109] found that the supplement of fish peptone to *Azotobacter vinelandii*
435 cells not only enhanced P(3HB) formation but lead to pleomorphic and osmotically sensitive

436 cells, for which fragility was exploited in a simple alkaline treatment. It is believed that the
437 addition of fish peptone stimulated P(3HB) formation at a much higher rate than cell protein
438 formation, impairing growth and causing partial cell lysis. Immersion of the biomass in 1 N
439 NH_3, at 45°C, for 10 min, was enough to yield a final product consisting of 94% P(3HB), 2%
440 protein, 4% non-protein residual biomass.

441 Divyashree and Shamala [110] also investigated the effect of the cultivation media on the
442 robustness of the cellular wall. They demonstrated that *B. flexus* cells grown on inorganic
443 nutrients lacked diaminopimelic acid in the cell wall and had a lower amino acid concentration
444 than cells cultivated on organic sources (yeast extract or peptone) and therefore could be lysed
445 more easily.

446 The exposure of halobacterial cells to low salt concentrations as a procedure for extracting
447 P(3HB) was patented by Escalona et al. [111]. Rathi et al. [112] used osmotic lysis in the
448 presence of an alkali or detergent as a simple mechanism for P(3HB) recovery from halophilic
449 bacteria. From 90% to full recovery and high purity (90%) was obtained regardless of using
450 wet or dry biomass. P(3HB-*co*-3HV) produced by the extremely halophilic archaeon *Haloferax*
451 *mediterranei* has been also extracted by osmotic pressure combined with a reduced amount of
452 chemicals (SDS and sodium hypochlorite) and little solvent (chloroform) [113,114]. A similar
453 treatment has been employed by Choi and Lee taking advantage of the specially fragile walls
454 of recombinant *E. coli* cells [61].

455 **2.6 Genetically induced cell lysis**

456 In an attempt to reduce the costs associated with DSP operations for the case of intracellular
457 metabolites, bacterial and yeast strains have been designed and constructed to secrete certain
458 fermentation products that naturally accumulate in the cytoplasm [115].

459 Lysis genes from bacteriophages have been introduced in bacteria producing scl and mcl-PHAs
460 [116–119]. Most bacteriophages have a holin-endolysin lysis mechanism, in which small
461 proteins (holins) oligomerize in the membrane creating holes that allow the endolysins to reach
462 the bacterial wall and degrade it. The time at which the holins cause the permeabilization of

the membrane can be 'programmed' based on culture conditions. Resch et al. [120] provoked cell lysis by a switch in temperature once the fermentation was over and Zhang *et al.* [121] forced the cell wall to collapse by transferring the cells from a solution rich in magnesium to a buffer with a lower magnesium concentration. A two-carbon source system was used to induce cell disruption in *B. megaterium* fermentation; in this case, the regulatory system was induced by xylose but inhibited by glucose. Once the latter carbon source was depleted, cells spontaneously liberated the accumulated PHA [122].

Sabirova et al. [123] presented an invention for extracellular production of PHAs through genetic modifications. *Alcanivorax borkumensis* SK2 was found to overproduce PHA when growing on alkanes, which resulted in extracellular deposition of the polymer. The inactivation of a particular enzyme rechannelled the intermediate metabolites of the alkane degradation towards PHA synthesis.

3 ADVANCED SEPARATION TECHNIQUES

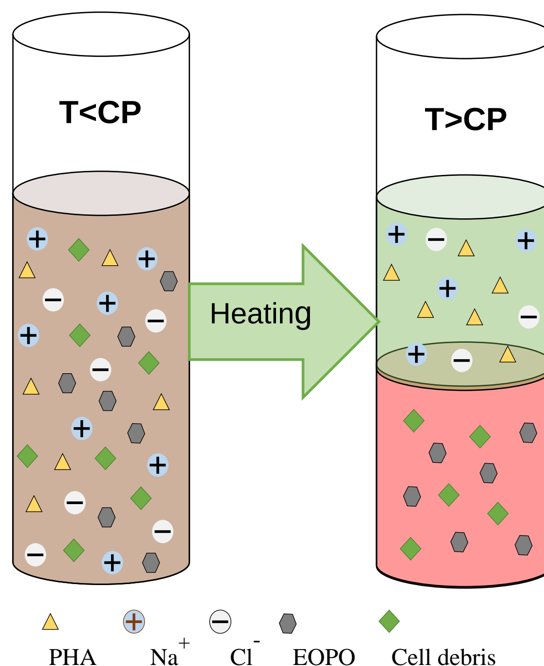
3.1 Aqueous two-phase systems

Aqueous two-phase extraction (ATPE) systems are based on the transfer of a solute from one aqueous phase to another phase. They can be of a polymer-polymer type or polymer-salt type. ATPE have been used for antibiotic, enzyme, nucleic acid and protein recovery and show unique features: they involve relatively safe and eco-friendly phase forming components (with a large aqueous base), they provide a rapid separation without energy input and can handle large capacities, which result in an ease of scalability [124].

Divyashree et al. [125] used an ATPS, containing polyethylene glycol (PEG) and phosphate, to separate PHA produced by *B. flexus* cells. Three pretreatment options to lyse the cells were explored and the ATPS performance, on the hydrolysed cells was compared with that of chloroform. The mixture of PEG (12%, w/v), potassium phosphate (9.7%, pH=8.0) and cell hydrolysate was left to separate at 28°C for 30 minutes. In the partition system, the PEG phase containing PHA was the upper phase while the residual cellular material was at the bottom. In the case of the enzymatic hydrolysis at 37°C and for 2 hours prior to the phase separation, the

490 protease, secreted by the *Microbispora* used, could be recovered in the PEG phase together
491 with PHA. Furthermore, the enzymatic pre-treatment yielded a higher molecular weight
492 polymer than the sodium hypochlorite method.

493 PEG was replaced by thermoseparating polymers, which can be more easily recyclable. They
494 consist of random, diblock, and triblock copolymers of hydrophilic ethylene oxide (EO) and
495 hydrophobic propylene oxide (PO), thus named as EOPO copolymers. Thermoseparating
496 polymers are soluble in water up to the lower critical solution temperature or cloud point (CP).
497 By raising the temperature, two phases are formed, i.e. the polymer and the waste phase. Leong
498 et al. [126,127] studied the influence of the molecular weight of the thermoseparating polymers,
499 their concentration, and the type of salt added to promote the partition of biomolecules to the
500 targeted phase, $(NH_4)_2SO_4$, K_2HPO_4 or NaCl. The authors acknowledged that the ATPS system
501 was not a total solution but could be a primary step for purification, which would benefit from
502 a volume reduction. The mechanism of separation of ATPS with thermoseparating polymers is
503 shown in Figure 2.

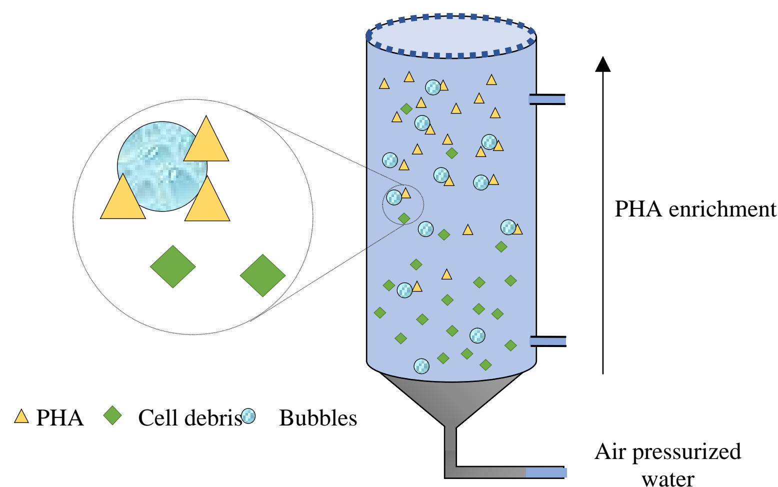


504

505 **Figure 2: Schematic representation of a thermoseparating polymer based two-phase system for the recovery**
506 **of PHA.**

507 ## 3.2 Air classification

508 This technique consists of the separation of finely ground solid particles based on size or
509 weight. The resulting finer fraction, with a high concentration of the product of interest, is later
510 recovered with 85-95 % yield and 85-90 % purity, using physical methods such as filtration or
511 centrifugation[128]. The first process patented by Procter & Gamble using air classification
512 involved fine grinding of the biomass originating particles smaller than 100 µm. These particles
513 were subjected to air classification and the fine fraction washed with an alcohol solution and
514 the solid pellet of PHA separated from the residual supernatant [129].
515 Van Hee and colleagues [130] carried out an in-depth study on the mechanism of flotation as a
516 separating mechanism of mcl-PHA granules from the cell debris of *P. putida*. They used a
517 flotation device with an enzyme treated broth, near the iso-electric point of bacterial debris and
518 inclusion bodies, and water injection. The samples recovered at the bottom and the top were
519 freeze dried. PHA content was determined by GC-FID. An 86 % purity was achieved, and the
520 authors pointed out that there was room to improve this value with continuous flotation, when
521 non-selective transport of particles in water is reduced. A schematic representation of the
522 dissolved air mechanism to separate PHA is presented in Figure 3.



523

524 **Figure 3: Representation of the separation of PHA from bacterial broth by selective air-dissolved flotation.**

525 # 4 PURIFICATION

526 The type of contaminants remaining in the recovered polymer depends on the extraction method
527 used. Lipids and colouring substances are often co-extracted when using non-polar solvents
528 while proteins are usually found when biomass has been chemically digested in an aqueous
529 solution. Some of the techniques presented above can be applied, repeated or combined
530 rendering a polymer suitable for a certain application [131].

531 The technology of PHA purification must satisfy strict requirements if the polymer is to be used
532 in the medical field. Biologically active contaminants that can trigger immunological reactions
533 need to be reduced to values that comply with United States Pharmacopeia policies.
534 Lipopolysaccharides (LPS), present in the outer membrane of Gram negative bacteria, act as
535 endotoxins and induce adverse effects when they are in contact with blood. Thereby, they
536 represent a serious concern and medical-grade PHA needs to be meticulously purified to get rid
537 of them. Repeated dissolution and precipitation of the polymer is common practice when
538 purifying this grade of polymer [132].

539 To further reduce the endotoxin content, hypochlorite and inorganic and organic peroxides
540 (hydrogen peroxide and benzoyl peroxide mainly) have been successfully used by Williams et
541 al. [94]. Horowitz and Brennan [133] patented a purification strategy based on ozone. In
542 addition to solubilisation of impurities, ozone has a bleaching and deodorizing effect on the
543 polymer and eliminates the hazards of operating with hydrogen peroxide and the polymer
544 degradation caused by sodium hypochlorite treatment. Zhang et al. [134] studied the endotoxin
545 removal capacity of an adsorbent of crystalline calcium silicate hydrate.

546 Wampfler et al. [135] improved the simultaneous extraction and adsorption-based purification
547 strategy developed previously in which a large loss of product was incurred. Mcl-PHA was
548 extracted from freeze-dried biomass, in the presence of activated charcoal and ethyl acetate for
549 1 h (solvent to biomass ratio of 15:1v/w). An activated charcoal to solvent ratio of 0.25 or 0.5
550 (v/v) was used depending on the type of polymer being extracted. Solids were eliminated by
551 pressure-assisted filtration after extraction. The filtrate was next passed through a filter cake

552 and then through a membrane to remove the finest coal particles. A 55 to 75% yield was
553 achieved using this optimized protocol.

554 5 FACTORS AFFECTING THE RECOVERY OF PHAs

555 As previously discussed, the recovery of PHAs is a complicated process that normally requires
556 more than a single unit operation. The selection of a suitable combination of methods depends
557 upon different factors [136]. It is crucial to specify the polymer quality requirements before
558 considering any DSP method. Mechanical and physical properties, such as molecular weight,
559 will be severely affected by the type of extraction method. Also the PHA natural form
560 (amorphous or crystalline) might change with certain treatments [137]. Depending on the target
561 purity and endotoxin level allowance, an additional purification step might be required.
562 The properties of the PHA produced will determine the compatibility with certain DSP
563 methods. For example, mcl-PHAs have a wider spectrum of solvents than P(3HB). Besides the
564 type of polymer, its content affects the integrity of the membrane. Based on the percentage of
565 PHA accumulated in the cell, the decision of solubilizing the PHA or the non-PHA cellular
566 matter (NPCM) should be made [4]. The density of the polymer and average size of the
567 granules finally limits the number of separation systems that can be used.
568 Lastly, the type of microorganism and culture conditions can also influence the DSP. Usually
569 PHA-accumulating wild type bacteria have stronger cell walls than recombinant strains [138].
570 Mixed microbial cultures (MMC) are claimed to be more resistant to cell hydrolysis than pure
571 cultures [44]. The cellular density of the culture affects the efficiency of the recovery process
572 too. Compared to heterotrophic bacteria, the DSP of algae and cyanobacterial cultures is
573 particularly difficult due to the lower biomass concentrations achieved in the fermentation
574 [139]. Remaining oily substrates can be an obstacle for PHA isolation and a degreasing step,
575 with an organic solvent or supercritical fluids, might be needed [140].

576 6 ENVIRONMENTAL CONSIDERATIONS

577 Bioplastics are presumed to present an ecological advantage with respect to conventional
578 plastics since they are derived from renewable sources. This needs to be thoroughly evaluated

579 by considering the environmental impact along the whole life cycle [141]. Results of the life
580 cycle assessment of PHA production are however controversial. Different indicators such as
581 global warming potential, carbon footprint, etc. and benchmarking against diverse products
582 have led to contradictory results, especially when fermentation substrates and energy systems
583 differ widely from one study to another. Furthermore, most studies are based on pilot-scale
584 data, which do not represent a real industrial production system [142]. However, it is important
585 to highlight the usefulness of LCA to pinpoint 'ecological hotspots'[143].
586 Fernández-Dacosta et al. [144] performed a LCA on three downstream strategies for PHA
587 recovery from MMC, namely alkali treatment (I), surfactant-hypochlorite (II) and solvent based
588 extraction (III), results are summarised in Table 4. The former was found to be the most
589 favourable from both, environmental and economic point of view. The surfactant-hypochlorite
590 method required an additional step and had a higher ecological footprint due to the usage of
591 chemicals (SDS). The distillation to recover and recycle DCM and ethanol in the solvent-based
592 approach incurred in the highest costs and environmental impact because of the high duties in
593 reboiler and condenser. Nonetheless, the polymer extracted by this route was the only one with
594 enough quality to be applied as a thermoplastic.

Table 4: Comparison of different strategies for PHA recovery, adapted from Fernández-Dacosta et al. [144]**.**

I	73.5	1.40	2.4	106	70
II	75.8	1.56	2.1	109	73
III	82.8	1.95	4.3	156	79

595

596 On another study, Righi et al. conducted a LCA of polyhydroxybutyrate extraction using
597 simulated industrial scale data using DMC and compared the results with those obtained with
598 1,2-dichloroethane. In all categories assessed (climate change, photochemical ozone formation
599 and ecotoxicity), DMC showed better environmental performance than the halogenated
600 hydrocarbon solvent. It was also found that the extraction applied to dry biomass resulted more
601 favourable than the one from slurry biomass [145].

602 **7 REMARKS**

603 Solvents have been the most explored alternative for PHA recovery and good results, in terms
604 of yield, purity and polymer quality, have been achieved with this method. Nevertheless, in
605 order to adopt the principle of producing a sustainable replacement for an oil-derived product,
606 green solvents and recycle loops need to be implemented. sCO_2 can be an interesting alternative
607 if the cost of production can be reduced; a biorefinery approach where the sCO_2 is produced in
608 the same facility could contribute towards this goal. Other promising solvents are DMC or ethyl
609 lactate derived from microbially produced lactic acid [146].
610 To overcome the limitation of the most commonly used chemical, i.e. sodium hypochlorite, low
611 concentrations of surfactant solutions, acids and bases can be employed which could result in
612 less environmental burden. Enzymatic hydrolysis could only compete with the chemical
613 approach if crude enzyme hydrolysates are produced. Mechanical systems, specially bead
614 milling, have many attractive advantages over solvents and chemicals, provided a clean energy
615 system is available.
616 Within the advanced separation techniques, ATPE appears as a cost-effective and scalable
617 method to be used as a first step in the purification process. The combination of operations must
618 be carefully evaluated based on the final product specifications but ultimately Life Cycle
619 Assessments should determine whether the selected route is a sustainable solution.
620 As already mentioned, DSP can contribute to a high share of the total costs. Developing less
621 expensive DSP operations is especially important if using MMC or starting with low value
622 carbon sources. Furthermore, the anaerobic digestion of NPCM in biogas plants or the
623 chemical/enzymic hydrolysis to a rich carbon and nitrogen source for subsequent microbial
624 cultivations can enhance the overall feasibility of the process [147].
625 As presented in this review, the suitability of an extraction technique is intimately linked to the
626 system characteristics and final use of the polymer, but it can never neglect the ultimate purpose
627 of the process: the production of green plastics.
628

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