sciendo

The EuroBiotech Journal

REVIEW

Bioprocess Engineering

Established and advanced approaches for recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from surrounding microbial biomass

Martin Koller[1,2*]

Abstract

Downstream processing for recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from biomass constitutes an integral part of the entire PHA production chain; beside the feedstocks used for cultivation of PHA-production strains, this process is currently considered the major cost factor for PHA production.

Besides economic aspects, PHA recovery techniques need to be sustainable by avoiding excessive use of (often precarious!) solvents, other hazardous chemicals, non-recyclable compounds, and energy. Moreover, the applied PHA recovery method is decisive for the molecular mass and purity of the obtained product, and the achievable recovery yield. In addition to the applied method, also the PHA content in biomass is decisive for the feasibility of a selected technique. Further, not all investigated recovery techniques are applicable for all types of PHA (crystalline versus amorphous PHA) and all PHA-producing microorganisms (robust versus fragile cell structures).

The present review shines a light on benefits and shortcomings of established solvent-based, chemical, enzymatic, and mechanical methods for PHA recovery. Focus is dedicated on innovative, novel recovery strategies, encompassing the use of "green" solvents, application of classical "PHA anti-solvents" under pressurized conditions, ionic liquids, supercritical solvents, hypotonic cell disintegration for release of PHA granules, switchable anionic surfactants, and even digestion of non-PHA biomass by animals.

The different established and novel techniques are compared in terms of PHA recovery yield, product purity, impact on PHA molar mass, scalability to industrial plants, and demand for chemicals, energy, and time.

Keywords: Biopolyesters; Biopolymers; Downstream processing; Green solvents; Ionic liquids; Polyhydroxyalkanoates (PHA); Supercritical solvents

[1]University of Graz, Institute of Chemistry, NAWI Graz, Austria

[2]ARENA (Association for Resource Efficient and Sustainable Technologies), Graz, Austria

*Corresponding author: M. Koller
E-mail: martin.koller@uni-graz.at

DOI: 10.2478/ebtj-2020-0013

© 2020 Authors. This work was licensed under the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 License.

Introduction

The application of polyhydroxyalkanoates (PHA), a group of microbial biopolyesters with diverse well-known (storage materials) and emerging (protectants against various stress factors) biological functions (1), to substitute petrochemical plastics in diverse fields of application is heavily discussed since decades, with several ups and downs on the way to their broad market penetration. This market penetration, however, still lags behind the high expectations of the scientific community (2). Despite the fact that these biopolyesters offer plenty of environmental advantages such as biocompatibility, biodegradability, compostability, and embedding in the natural carbon cycle, the major obstacle for the market success of PHA are still their production costs. Economically, PHA are not yet competitive with mineral oil-based plastics from petro-chemistry, which are produced by rather simple chemical processes established since many decades. As biological, intracellular products of the secondary metabolism of many bacteria, archaea, and a limited number of yeasts, volumetric productivity of PHA can intrinsically not become competitive with petrochemical plastics due to the limits set by Nature. This can be visualized by

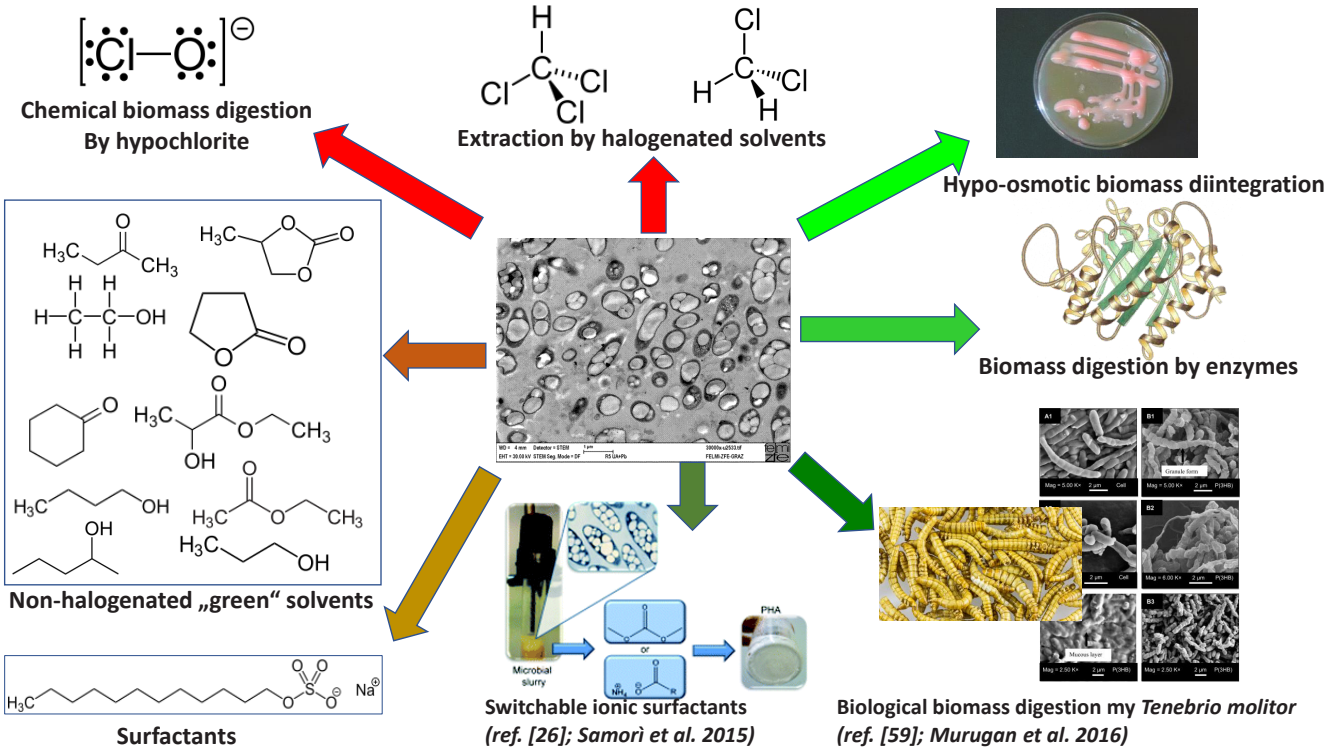

Figure 1. Overview of established and emerging PHA recovery techniques.

the fact that kinetics of petro-plastic synthesis can be boosted by applying high temperature and pressure regimes and often toxic catalysts, while biosynthetic PHA formation occurs under ambient or, in the case of extremophiles, under only slightly elevated temperature and pressure. Considering the fact that PHA biosynthesis constitutes an aerobic process, the same goes for substrate-to-product conversion: yields are limited by the indispensable loss of carbon source by oxidative degradation of substrate, generating CO_2 during cellular respiration, while excellent product yields are typical for petrochemical plastic production (3).

In the meanwhile, considerable progress was made in terms of reducing the costs directly associated to the processes of biomass growth and subsequent PHA accumulation inside the cells. In this context, costly organic carbon sources were replaced (at least on laboratory scale) by carbonaceous waste and surplus materials from diverse industrial branches, especially from agro- and food industry (4), and attempts were made in converting inexpensive gaseous substrates like CH_4, CO_2, or syngas towards biomass and PHA by microbial specialists like class II methanotrophs (5,6), cyanobacteria (7), and pink *Rhodospirilli* (8). Moreover, the new concept of "Next generation industrial biotechnology" (NGIB) builds on the use of extremophilic production strains, which can be cultivated at minimum energy and sterility precautions in open bioreactor facilities, and which are conveniently accessible towards genetic modification to enhance their biocatalytic performance (9). In the field of PHA production, this concept was already realized for halophilic, genetically tailored strains from the genus *Halomonas* (10). As another trend, we currently witness dynamic development in the use of mixed microbial cultures (MMCs) for production of PHA coupled to mitigation of pollutants from waste water; here, quality control of generated PHA still is a remaining issue to be solved (11).

Beside the mentioned aspects of substrates, energy requirement, and bioengineering, it is currently undisputed that downstream processing, needed to recover intracellular PHA from the surrounding non-PHA cells mass, significantly contributes to the entire PHA production costs, hence, the smart choice of a recovery method has a dominant impact on the overall process economics. Moreover, appropriate downstream processing is a major factor for the ecological footprint of microbial bioplastics (12). Issues like the type and amount of extraction solvents, energy input, recyclability of solvents and other chemicals, and water requirement are factors determining the economic and environmental feasibility of a given PHA recovery process, and must be weighed against to obtained product quality and recovery yield (13). The review at hand provides an updated overview of established and emerging techniques for PHA recovery, with focus dedicated to trends and progress observed during the recent years years (for an overview, see **Fig. 1**).

Downstream processing in PHA production

After stopping the biotechnological PHA production process (cultivation of living cells in bioreactors), typically by stop of aeration and pasteurization, PHA-rich biomass needs to be separated from the supernatant, hence, the spent fermentation broth. This separation is typically accomplished by means of sedimentation, flocculation, or centrifugation, or, to a lower extend, by filtration techniques. While biomass recovery nor-

mally is accomplished batch-wise, it can also be performed by continuously operated separators in case of continuous cultivation processes. After this separation step, PHA-rich biomass is typically dewatered (thermal drying step or lyophilization), and appropriate techniques are needed to recover PHA from dry microbial biomass in a short time, at high yields, without negatively impacting polymer quality (especially molecular mass), and by environmentally benign approaches.

Economics of PHA extraction highly depend on available equipment, energy and chemicals demands, on PHA recovery yields and recyclability of the applied compounds. Briefly, solvent-based extraction methods and cell disruption by chemical, enzymatic, or mechanical techniques, or combinations thereof, are described, as it is broadly dealt with in many recent review articles. However, when carefully reading these publications, it becomes obvious that every one of these recovery methods has disadvantages, either economically, ecologically, for safety aspects, disappointing recovery yields, mediocre product purity, or inadequate scalability (13-16). Notably, best-established PHA recovery methods, which generate superior recovery yields and maximum product purity, are based on extraction techniques using noxious halogenated solvents, predominately chloroform, in other words, materials that should not play a role in a sustainable production chain anymore. Consequently, alternative PHA recovery methods are presently in the development stage; these methods resort to fundamentally different approaches:

Extraction of PHA from biomass

Halogenated solvents as the bench mark for the extraction performance of PHA solvents

Solvent-based extraction methods, mainly those using chloroform or, to a minor extent, dichloromethane, typically deliver excellent extraction yields and high polymer purity, as it was reported already years ago in a detailed study by Ramsay and colleagues (17). Yet, these processes produce extreme volumes of solvents, which are often detrimental for the environment and human health. In addition, chloroform, the most often used PHA extraction solvent, is highly irritating for mucous membranes, the respiratory tract and eyes; moreover, it is supposed to be carcinogenic. Besides, many outstanding PHA solvents stem from petrochemistry. Furthermore, solvent-based PHA extraction typically destroys the natural characteristics of PHA granules by reducing molecular mass via random and chain-end scission, particularly at higher temperature and prolonged extraction time; this can obstruct their further processing. In addition, after dissolving PHA in halogenated solvents, a precipitation step is generally required to obtain PHA of high purity (simply evaporating the solvent results in impurity inclusions in the polymer); for this purpose, "PHA anti-solvents" such as ethanol, acetone, methanol, hexane or heptane are typically provided in excess and under cooling to a concentrated PHA solution in the halogenated solvent to drastically reduce PHA´s solubility. After this precipitation, a mixture of PHA solvent and PHA "anti-solvent" is left, which can be separated into the original compounds only under excessive energy expenditure, typically via distillation or rectification, which makes the solvent recycling uneconomic (17).

Nevertheless, due to its convenient use on laboratory scale, this method is till today frequently used for PHA recovery, as only recently shown by Rebocho *et al.*, who co-cultivated the strain *Curpiavidus necator* DSM 428, a producer of crystalline short-chain-length (*scl*-) PHA, and *Pseudomonas citronellolis* NRRL B-2504, a producer of a highly amorphous medium-chain-length (*mcl*-) PHA bio-latex. Co-cultivation of both strains and co-extraction of the fundamentally different types of PHA resulted in recovery of a natural bioplastic blend, consisting of about 48 wt.-% *scl*-PHA (the homopolyester poly(3-hydroxybutyrate) - PHB) and 52 wt.-% *mcl*-PHA, consisting of 3-hydroxydecanoate (3HD), 3-hydroxyoctanoate (3HO), 3-hydroxydodecanoate (3HDD), and 3-hydroxytetradecanoate (3HT). For this purpose, authors centrifuged the cultivation broth harvested from the bioreactor. The cell pellets obtained this way were resuspended in deionized water and centrifuged again. The obtained biomass pellet was then lyophilized for removal of water, and PHA was extracted from the dried biomass via Soxhlet extraction with chloroform at 80 °C for 48 h. The pure polymer blend was isolated by precipitation in the ten-fold volume of ice-cold ethanol under continuous stirring (18).

Another recent study by Ojha and Das described PHA biosynthesis by the halophilic yeast *Pichia kudriavzevii* VIT-NN02 from agricultural waste materials, namely banana peels as carbon source, and chicken feathers hydrolysate as nitrogen source. Here, it should be emphasized that PHA production by eukaryotes like yeast is a rather rare phenomenon. The obtained PHA was identified as a *scl*-PHA, specifically a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) copolyester, consisting of 57.5% 3-hydroxybutyrate (3HB), and 42.5% 3-hydroxyvalerate (3HV) units. PHA extraction was again performed by the chloroform technique. After the cultivation, cells where harvested by centrifugation, and the obtained pellets were washed twice with deionized water and air-dried at 55 °C. The dried biomass (1.0 g) was stirred in a mixture of 7 mL chloroform and 3 mL aqueous NaOCl solution (4%), which also constitutes a chlorinated product. After stirring, the mixture was centrifuged, and three separate layers were obtained. The PHA dissolved in chloroform (the lowermost layer) was precipitated by adding the nine-fold amount of ice-cold methanol as white, highly pure (99.99%) product, which was separated by centrifugation (19). Reports in references (18) and (19) already visualize the excessive quantities of organic solvents accruing by these solbent-"anti-solvent" extraction methods.

When using ethanol as PHA "anti-solvent" to precipitate PHA from chloroform solution as described by Rebocho *et al.* (*vide supra*), a one-phase mixture of the alcohol with the PHA solvent chloroform is generated. Separation of this mixture is energy demanding, and therefore economically hardly feasible. However, recycling of chloroform from this mixture becomes possible in a convenient, not energy demanding process by

simply adding appropriate amounts of water. At the right chloroform-water-ethanol ratio, a ternary 3-components-2-phases system is created, which consists of a high-density chloroform phase of about 95% purity containing ethanol residues as stabilizers like it is also the case in commercial chloroform, which can be recycled and used for subsequent extraction cycles. The second phase, a low-density ethanol/water mixture contains only minor quantities of chloroform (20). However, the author of this review is not aware of any implementation of this approach on a pilot or industrial scale PHA production.

"Green" solvents

General

Approaches to improve PHA extraction from biomass use less toxic, "green" solvents like various alcohols (ethanol, 1-propanol, 1-butanol, etc.) (21), ketones (acetone (22), cyclohexanone (23), methyl ethyl ketone (MEK) and methyl isobutyl ketone (MIBK) (24,25)), dimethyl carbonate (26), open or cyclic esters (acetic acid esters, lactic acid esters, γ-butyrolactone (GBL), etc.) (23), THF (27), or cyclic carbonates (28). Some among such halogen-free "green" solvents, such as acetone, diverse alcohols, acetic acid, lactic acid, etc., can be produced biotechnologically from renewable resources, while others, like GBL, cyclohexanone, or THF, are products of petrochemistry. Some among these solvents, such as cyclic and linear carbonates, need high temperature for efficient PHA extraction, which, beside being energy-demanding, typically reduces the biopolyesters´ molecular mass, in particular when transesterification between esters used as solvents and PHA takes place. In this context, reduced molecular mass disadvantages the further processability of isolated PHA towards vendible bioplastic items (17). Importantly, the PHA-solvation potential of these "green" solvents highly depends on the crystallinity of the biopolyester, hence, amorphous *mcl*-PHA is more easily dissolved by such solvents than crystalline *scl*-PHA.

Acetone and other non-cyclic ketones

As an example of such novel solvents, acetone is a volatile and easily recyclable compound. When used for PHA extraction, highly pure products are obtained, without significant reduction of molecular mass, which makes this ketone an auspicious candidate for PHA recovery. As mentioned, it should be noted that not all "green" solvents like acetone are suitable to dissolve all types of PHA; importantly, it is generally no simple task to predict solubility of different PHA macromolecules in a given solvent without testing it experimentally. As recently demonstrated by Cerrone *et al.*, who extracted an amorphous PHA copolyester consisting of 3HDD, 3HD, 3HO, and 3-hydroxyhexanoate (3HHx) (poly(3HDD-*co*-3HD-*co*-3HO-*co*-3HHx)) from lyophilized *Pseudomonas chlororaphis* biomass, such low-crystalline *mcl*-PHA is readily extracted by acetone even at room temperature. This is in contrast to conditions needed to dissolve *scl*-PHA like PHB homopolyester in acetone; here, temperature conditions above the solvent´s boiling point are required (29). This matches a study by Asrar and co-workers from 2000; these authors mentioned the lacking solubility of *scl*-PHA in most non-halogenated solvents, including the typical "PHA-antisolvent" acetone, under conditions normally used for PHA extraction on an industrial scale (temperature far below the solvent´s boiling point, no elevated pressure) (30).

However, one can profit from the different solubility of *scl*- and *mcl*-PHA in acetone to fractionate natural blends consisting of *mcl*- and *scl*-PHA, which might occur in a microbiological sample, e.g., in MMCs. This was recently demonstrated by Rebocho and colleagues, who, as described in section 3.1, co-extracted a natural PHB-*mcl*-PHA blend by using chloroform. To separate PHB and the *mcl*-PHA fraction, the latter was dissolved in acetone at 30 °C, while PHB remained as non-soluble solid. Practically, 1 g of the co-extracted polymer blend was constantly shaken with 30 mL of acetone at 30 °C for 24 h. The acetone-soluble *mcl*-PHA fraction was then conveniently separated by centrifugation from the acetone-insoluble PHB fraction, and predominantly consisted of 3HD and 3HO, beside minor amounts of 3HDD, 3HTD, and traces of 3HHx (18).

For *scl*-PHA extraction by acetone under high temperature and pressure, Koller *et al.* developed a convenient aluminum device for PHA extraction consisting of an extraction-, filtration-, and precipitation unit. This device was successfully applied to recover a PHA heteropolymer from lyophilized biomass of the haloarchaeal strain *Haloferax mediterranei* under high temperature and pressure by using acetone as the sole solvent. In this context, it should be noted that, under ambient conditions for temperature and pressure, acetone does not dissolve crystalline *scl*-PHA like PHB or PHBHV. However, under conditions of about 120°C and 7 bar, it was possible to extract PHA under continuous stirring in the extraction unit of the device. By opening a valve, the mixture consisting of PHA and lipids dissolved in acetone and solid non-PHA cell material was passed from the extraction- to the filtration unit, where non-PHA cell material was retained. Finally, PHA was precipitated from solution in the final precipitation unit simply by cooling to room temperature. Importantly, the entire system, before being pressurized, was floated with nitrogen gas to remove oxygen in order to prevent the risk of explosion. Reported recovery yields and product purity were competitive with results obtained for parallel control setups with the "benchmark solvent" chloroform; purity for chloroform extraction in batch setups at room temperature amounted to 97.7%, Soxleth-extraction by chloroform yielded a purity of 99%, while the new acetone process delivered PHA of 98.4% purity. Extraction yields for the different methods were also in a similar range (96.8%, 98.9%, and 91.6%, respectively). During optimization of this process, it also turned out that this organism accumulates a natural PHA blend; part of this material (with high fraction of monomers different from 3HB) was shown to be soluble in acetone even under Soxleth-extraction conditions, while the major part of the polymer required the high temperature conditions prevailing in the extraction apparatus (22).

Similar experiments resorting to ketones for *scl*-PHA ex-

traction were later carried out by other authors. Based on the fact that ketones like MIBK and especially MEK can dissolve P(3HB-*co*-3HHx) copolyesters, both from wet and dry biomass (24), Yang *et al.* studied the application of MEK as a solvent to extract PHBHV produced by different wild type and recombinant strains. The extraction was carried out at 100 °C for 5 min; similar to acetone application, a gel was formed after onset of cell lysis. A recovery yield of 93% and a purity of 91% were obtained, which is somewhat lower than in above reported acetone process. Since MEK has a boiling point of only 79.6 °C, this extraction was also carried out under pressurized conditions (transparent closed glass vials to allow observation of polymer dissolution). According to the authors, the considerably higher boiling point of MEK (79.6°C) in comparison to acetone (56.5°C) makes this ketone less dangerous in terms of explosion due to excessive pressure generation. Moreover, the low density of MEK (0.8 g/mL) allows convenient separation of cell debris (density about 1.1 – 1.3 g/mL) from the PHA solution via centrifugation or even just by sedimentation. This is a considerable advantage to chloroform, where a filtration step is needed to separate cell debris from the polymer solution. However, precipitation of the polymer from MEK solution (which results in higher product purity and easier operation conditions than MEK evaporation) needs the excessive addition of alkanes like n-hexane, which requires a subsequent distillation for solvents separation. In the case of acetone, solvent-PHA separation is simply accomplished by cooling down the solution (22). In addition, MEK-extracted PHA had a lower polydispersity (Đ) than PHA chloroform-extracted in parallel setups, which indicates that MEK only extracts a certain fraction of PHA present in the biomass sample (25).

Alcohols

Another example of non-halogenated PHA solvents resorts to the biogenic distillation by-products from ethanol production (fusel oil containing 1-propanol, iso-butanol, *(S)*-2-methyl-1-butanol, and isopentanol). This fusel alcohols are generated as by-product in an integrated production process for cane sugar, bioethanol, and PHA by the Brazilian company PHB/ISA, hence, they are available in-house at zero cost in this company. Looking carefully at what is described by this company about the process, it however looks quite complicated: Cultures of *C. necator* or *Burkholderia* sp., after the fermentation, are thermally inactivated, flocculated, and concentrated to a cell slurry of up to 300 g/L density. Now, this slurry undergoes a multi-step extraction process with fusel oil in continuously stirred tanks. From the extract, cell debris needs to be removed, and, by cooling down, the PHA solution turns into a gel. By pressing and evaporation, the major part of the solvent is removed. From the remaining polymer, residual fusel oil is removed by dispersion in water, and subsequent distillative recovery of the solvent. After vacuum drying, the polymer can finally be processed to a PHA granulate. Purity of the obtained polymer is reported to be in a similar range (about 98%) like purity obtained by established extraction methods based on chloroform (31).

In the context of bioethanol production, the direct use of the "green", easily recyclable solvent ethanol was only recently demonstrated by Garcia and colleagues. These authors were able to prepare PHB homopolyester produced by an *Azotobacter vinelandii* mutant strain with a weight average molecular mass (M_w) of more than 6,000,000. The centrifuged wet biomass (no drying step required!) was resuspended in distilled water, centrifuged again, and, by vortexing, the biomass was resuspended again in ethanol. This suspension was stirred and heated close to ethanol´s boiling temperature in a closed flask for 30 min. This is in contrast to above-described acetone-extraction process (3.2.2), which runs at a temperature far above the solvent´s boiling point. Centrifuging the obtained mixture resulted in precipitation of a white pellet, which was purified by resuspension in acetone, centrifuged again, and finally dried at room temperature. This ethanol-based process gave a recovery yield of 85%, and a product purity of 95%. Surprisingly, even slightly lower purity and recovery yield were obtained in comparative experiments using the same biomass charge; these experiments were based on a combination of the halogenated compounds chloroform and NaOCl. Here, wet centrifuged biomass was resuspended in a mixture of chloroform and NaOCl solution, which generates a three-phase system; this system was mixed by vortexing and agitated for a longer time (20 h). After that, the high-density organic phase (PHA dissolved in chloroform) was recovered, and PHA was precipitated by adding excess 2-propanol as "PHA anti-solvent". Surprisingly, the obtained product had a M_w of only about 500,000, which is drastically lower than for the ethanol-extracted product (32).

Additional non-halogenated solvents

Other non-chlorinated solvents, namely the cyclic ketone cyclohexanone and the cyclic ester GBL, were studied by Jiang *et al.* as potential non-halogenated solvents to extract PHA from bacterial biomass, encompassing a detailed study of extraction kinetics in dependence on temperature. The strain *Cupriavidus necator* H16, cultivated on vegetable oils as carbon source, was used in this study as PHA production strain, and vegetable oil acted as carbon source. For experiments using cyclohexanone as solvent at 120°C, 95% of the total PHA content was extracted from biomass within 3 min; the product had a purity similar to that obtained using chloroform in parallel control setups. Lower temperature significantly decreased the extraction yield. Using the same temperature, GBL resulted in considerably lower recovery yields (only 50% recovered after 3 min) than when using cyclohexanone. For both solvents, molar mass and molecular mass distribution (Đ) of PHA were in the same range like products chloroform-extracted in parallel setups. Comparing product purity in dependence on extraction solvent, PHA contamination by nitrogen-containing residues was only slightly higher when the biopolymer was extracted by the two novel solvents compared to the reference case (chloroform). In this study, especially cyclohexanone took for the first time the stage as an auspicious candidate for more sustainable, halogen-free PHA recovery processes especially due to the expedient recov-

ery yields achieved; however, it should be emphasized that it constitutes a product of petrochemistry, and it is not accessible via means of biotechnology (23).

Recently, the use of "green" solvents butyl acetate, ethyl acetate, isoamyl alcohol, and 1,2-propylene carbonate for PHA recovery were compared by Gahlawat and Kumar Soni. The copolyester PHBHV with a 3HV fraction of about 25%, produced by *C. necator* from crude glycerol as carbon source, was extracted in these experiments. For 1,2-propylene carbonate, extraction was carried out by heating biomass with the solvent in screw cap bottles, and filtering the hot solution. The polymer was precipitated by adding the double volume of the *scl*-PHA "anti-solvent" acetone. Treatment with 1,2-propylene carbonate for 30 min at 120 °C resulted in a recovery yield of 90 % and a product purity of 95 %. while parallel setups with chloroform resulted in a recovery yield od 95%, and a product purity of 96%. This matches previous reports by these authors, which emphasize the excellent recyclability of this solvent, and the low risk associated to it due to its high boiling point of 242°C (34). Ethyl acetate extraction at 100 °C (by far exceeding the solvents´ boiling point of 77°C) achieved a recovery yield of 96 % and product purity of 93 %. PHA extracted by ethyl acetate was precipitated by adding the double volume of n-heptane. In direct comparison, ethyl acetate was more efficient for PHBHV recovery from biomass than the structurally related ester butyl acetate (33). This matches previous findings by Riedel *et al.*, who reported high product recovery yields and high product purities (up to 99%) when using ethyl acetate for extraction of dry biomass (24). Importantly, in Gahlawat and Soni´s study, recovery yield, purity, and Đ highly depended on the incubation time and temperature. Moreover, endotoxins, a group of inflammatory lipopolysaccharides produced by Gram-negative organisms like *C. necator*, but not by Gram-positives like *Bacilli*, were readily removed to a level below 5 EU per g PHBHV when treating with 2.5 N NaOH for 6 h; this level is low enough for biomedical (*in vivo*) applications of the products, where the endotoxin level is a critical factor (33).

Dimethyl carbonate

Among novel solvent-based techniques described for extraction of different types of *scl*-PHA, the use of the acyclic alkyl carbonate dimethyl carbonate (DMC), a fully biodegradable green solvent with low toxicity for human health and the environment, was described by Samorì *et al.* DMC is not only completely biodegradable, but also less harmful to human health and the environment than chloroform as the traditional "gold-standard" solvent. This process can either be used to extract PHA from dried biomass, or can even be directly added to concentrated bacterial fermentation broth. Its application results in reasonably high polymer recovery yields exceeding 85%, and generates products of outstanding purity higher than 95%. In both cases (dry biomass or wet fermentation broth), the extraction does not result in degradation of PHA´s molecular mass. Practically, lyophilized *C. necator* biomass rich in the homopolyester PHB was treated for 4 hours at the solvent´s boiling point (90°C) with DMC and, for comparison, at 50°C with the halogenated compound CH_2Cl_2. PHA recovery from solution was either accomplished via solvent evaporation or precipitation by adding cooled ethanol. The achieved PHA recovery yield using CH_2Cl_2 (98±3%) was in a slightly lower range as obtained with DMC (88±6%) (26).

In the context of DMC, de Souza Reis and colleagues (2020) used sludge from municipal wastewater treatment systems, which contained MMCs capable of PHA biosynthesis. Here, it should be noted that MMCs are typically highly robust microbial consortia with intracellular PHA fractions often only in the lower to medium range, which makes PHA recovery intrinsically more complicated. For this reason, MMCs are less prone to cell hydrolysis than pure cultures, where cell fragility can be further increased by means of genetic engineering and high intracellular PHA fractions. In this study, DMC was tested as a PHA extraction solvent from MMCs at different extraction times and biomass-to-solvent ratios. Overall, only a very small difference was observed when comparing the different extraction scenarios (extraction duration and ratio). An average product extraction of 30.7 ± 1.6 g of PHA per 100 g of biomass was achieved, which was in the same range as for comparative setups using chloroform and CH_2Cl_2. Further, 1-butanol was tested for purifying the obtained PHA samples at different treatment durations and PHA-to-solvent ratios under reflux. 1-butanol was selected because, similar to above discussed solvents MEK or fusel oil, it generates a gel with PHA at cooling, which allows a simple separation process. After purification with 1-butanol, a visible difference was observed for PHA samples obtained by the different tested scenarios (whitest product obtained after 0.5 h treatment at a PHA-to-solvent ratio of 1/100), although the measured purity (determined via TGA) of the obtained samples did not differ significantly. The overall purity after 1-butanol treatment increased from 91.2 ± 0.1% to 98.0 ± 0.1% (35).

Use of supercritical solvents

The advantage of supercritical fluids as solvents arises from their excellent solvation power, which is in a range similar to the solvation power of liquids; this is combined with supercritical fluids´ expedient diffusion power (lower density and viscosity than liquids) similar to gases. Supercritical CO_2 (sCO_2), which exits above CO_2´s critical point of 304 K and 74 bar, constitutes the best studied supercritical fluid; after the extraction, it simply evaporates as gaseous CO_2, not leaving any solvent restudies as it is the case for above discussed liquid solvents used for PHA extraction. Supercritical fluids definitely constitute an emerging group of compounds for extraction of many marketable compounds like caffeine, cholesterol, flavors, compounds present in hops, vitamins, hormones, and high-value oils; more recently, they were also successfully tested for PHA extraction from microbial biomass (36).

sCO_2 was applied to test PHA extraction from biomass of different types of microbial PHA production strains, with somewhat opposing conclusions. In this context, it was concluded by

Hampson and Ashby that sCO_2-mediated recovery of *mcl*-PHA by *Pseudomonas* sp. biomass requires some additional amounts of chloroform (37), which is in accordance with the results of Williams and colleagues, who reviewed the application of sCO_2 and other supercritical solvents as mixture with modifiers (organic solvents like alcohols), which helps to overcome the different polarity of CO_2 and the product to be extracted (38), and increases CO_2 solubility in the aqueous intracellular compartments (39). This matches the outcomes of a study by Hejazi and colleagues, who also achieved *scl*-PHA recovery yields of almost 90%, but only when mixing sCO_2 with methanol as modifier. In this study, PHB homopolyester produced by *C. necator* was extracted. The optimum conditions for cell disintegration and PHB recovery were determined with an exposure time of 100 min, a temperature of 40 °C, a pressure of 200 atm, and an addition of 0.2 mL methanol to 4 mL CO_2 (40). In contrast, a follow-up study by this research group demonstrated that, after appropriate alkaline pre-treatment of *C. necator* biomass, even highly crystalline *scl*-PHA can be recovered from lyophilized biomass by sCO_2 at higher yield than when adding highly modifiers (methanol or acetone). When using the non-polar modifier toluene, however, PHA recovery yields were increased due to solubilization of cell membrane components by this solvent. In total, 200 bar of pressure, 30 °C temperature, and 1 vol.-% of toluene with two times $scCO_2$ pressure release turned out as the optimum extraction conditions. Importantly, these authors found out that also wet biomass can be utilized to recover PHB by sCO_2 in order to save drying costs; no negative effect on molecular mass was observed compared to dry (lyophilized) biomass; however, product purity was slightly lower than when using lyophilized biomass (39).

Use of ionic liquids

The application of ionic liquids (ILs) for PHA recovery is a novel field in biopolymer research. ILs can be considered as "molten salts", having melting points lower than100 °C, often even below room temperature. These intriguing materials have special properties, superior to those reported for classical organic solvents, such as high thermal stability, insignificant vapor pressure, low flammability, and expedient ionic conductivity (41). While hydrophobic ILs can be applied for extraction of materials by liquid-liquid biphasic systems (42), highly hydrophilic ILs performed well in dissolving hardly soluble polymers like crystalline cellulose. In this context, Fujita and colleagues have reported the direct dissolution of wet (95% water content!) biomass of the cyanobacterium *Synechocystis* sp. using the hydrophilic ionic liquid 1-ethyl-3-methylimidazolium methylphosphonate ($(C2_{mim})(MeO(H)PO_2)$); no heating or pretreatment was needed. In this study, the authors for the first time hypothesized that intracellular products like PHA could successfully be recovered by ILs (43). As a follow-up, Kobayashi and colleagues studied $(C2_{mim})(MeO(H)PO_2)$ and other ionic liquids for removing the non-PHA part of the biomass of the cyanobacterium *Synechocystis* sp. PCC 6803 biomass explicitly with the aim to release intact PHB homopolyester granules. Especially $(C2_{mim})(MeO(H)PO_2)$ turned out to dissolve non-PHA materials, but leaving PHB granules intact. PHB, after dissolution, was separated and recovered by a simple filtration step. More than 98 % of PHA was recovered by this convenient process. Moreover, the authors emphasized the expedient recyclability of the solvent (44).

Removing the non-PHA part of biomass from PHA granules by enzymatic and chemical means

Enzymatic methods

Currently, we witness a paradigm shift away from extraction of the product (PHA), which accounts for up to more than 90% of the total dry biomass. In an increasing number of laboratories, it is recognized that removing the non-PHA part of biomass (often less than 10% of total dry mass) can be of economically higher efficiency. However, chemical methods used for this purpose are often expensive and resort to harmful or corrosive, hardly recyclable chemicals (sodium hypochlorite, strong bases or acids, or the irritating anionic surfactant sodium dodecyl sulfate (SDS)), while disintegration of non-PHA cell biomass for release of native PHA granules by green biocatalysts (enzymes) is not yet cost-efficient due to long time demands caused by the typically low reaction rates of enzymes, and leads to low product purities, as shown by industrial-scale tests (31). For enzymatic cell disintegration, which was already developed decades ago for industrial scale PHA recovery by Imperial Chemical Industries, UK (ICI), enzyme mixtures containing proteases, phospholipases, lysozyme, and nucleases were typically applied, often in combination with surfactants and the need for chemical purification of extracted PHA with strong oxidants like hydrogen peroxide (45). A novel enzymatic approach for digestion of non-PHA biomass was presented by Kachrimanidiou and co-workers. These authors prepared a crude enzyme cocktail via solid state fermentation (SSF) of the fungus *Aspergillus oryzae*. This enzyme mixture was applied to lyse *C. necator* cells for PHA recovery. Temperature and pH-value were optimized for maximum *C. necator* lysis, which reached about 90%; PHA purity and the recovery yield amounted to 97% and 98%, respectively (46). Future developments in this direction might go towards immobilized enzymes, which potentially might lower the economic impact of enzymes use.

Chemical methods

Marudkla and colleagues presented an advanced chloroform-free PHA recovery approach using the anionic surfactant SDS and sodium hypochlorite (NaOCl); optimized by an experimental Taguchi-design, the authors described the optimal conditions for the maximum PHA recovery (78.7%) when using 0.5% w/v SDS combined with 6% v/v NaOCl. Nevertheless, one should not neglect the fact that SDS is an irritating compound, while NaOCl is also a halogenated compound, which contradicts the aim of abolishing chlorine from bioplastic production (47). In addition, it was reported before that the application of NaOCl results in molecular mass reduction and generation of various halogenated compounds

(reaction products with cell constituents). Even when optimizing the digestion time and pH-value of the NaOCl solution, M_W of PHA was halved from 1,200,000 to 600,000, while Đ increased from 3 to 4.5 (48).

In the context of SDS, it should be noted that the use of surfactants for PHA recovery definitely has some advantages: similar to DMC (see 3.2.6), the surfactant can be directly added to high-cell-density cultivation broth, which saves efforts and costs for biomass dewatering and drying. Moreover, such surfactants do not result in degradation of PHA molecular mass. As downside of the metal, typically high amounts of surfactants are needed for PHA recovery, leading to excessive waste water formation, which in turn needs to be treated. Moreover, purity of PHA isolated merely by using SDS is generally mediocre, which typically demands additional purification steps (often carried out with halogenated solvents!), as exampled in a recent study by Mahansaria *et al*., who centrifuged biomass of the haloarchaeon *Halogeometricum borinquense* cultivated on glycerol as carbon source. The obtained cell pellet was suspended for 48 hours in 0.1% aqueous SDS solution in order to start lysis of cells. The lysed cell suspension was centrifuged again, and the remaining pellet was again subjected towards a washing step with 0.1% SDS solution, followed by washing with water. To remove any associated impurities, the obtained pure white pellet was washed in an 1:1 acetone:ethanol solution, and oven-dried at 80°C until reaching constant mass. The dried pellet was dissolved in boiling chloroform under reflux and filtered to separate eventually remaining undissolved matter. Chloroform was then evaporated in a hot water bath, and a thin PHA film was obtained (49).

In the study by Gahlawat and Soni (*vide supra*), also linear alkylbenzenemsulfonic acid was used as surfactant for recovery tests of PHBHV from *C. necator* biomass. At pH-value 5 and a temperature of 80°C, a maximum recovery yield of 80% was achieved, which is lower than parallel setups with ethyl acetate or 1,2-propylene carbonate, while product purity (90%) was also lower than when using the solvents (33).

Switchable ionic surfactants

Another novel technique applicable to wet biomass slurry, for the first time reported by Samorì *et al.*, applies salts of longer fatty acids (carboxylates) as surfactants. These materials disrupt cell membranes, and PHA granules are released. This process leads to outstanding polymer recovery yields exceeding 99%, and a purity of more than 90%. Among tested surfactants, especially ammonium laurate can be conveniently used as a so called "switchable anionic surfactant", meaning that it undergoes reversible conversion from a neutral water-insoluble form to a polar anionic water-soluble form via a pH shift, which allows convenient recycling; *in praxi*, it can simply be turned into the water-insoluble protonated form by adding CO_2. Precisely, a microbial slurry of lyophilized *C. necator* biomass and ammonium laurate was created at pH-value 10 (anionic form of the surfactant) to dissolve non-PHA cell material; now, PHA precipitates and can be collected via centrifugation. In a second step, the supernatant (containing laurate and cell debris) is neutralized by adding CO_2, which converts laurate into lauric acid, the non-water soluble, protonated form, which again precipitates and can be separated via centrifugation and recycled. What remains is a solution of dissolved cell constituents and NH_4HCO_3, which can be used as nutrient source for follow-up cultivations of microorganism (26).

In the context of ammonium laurate, a new protocol for PHA extraction from MMCs with high PHA-accumulating capacity was proposed by Mannina and colleagues. The MMC was enriched in a sequencing batch reactor (SBR) fed with a synthetic effluent mimicking fermented oil mill wastewater (OMW). Among all tested processes, the highest recovery yield and purity (74 ± 8% and 100 ± 5%, respectively) was obtained when using ammonium laurate (parallel setups used SDS as surfactant). Separation of the cell pellet was done via centrifugation and subsequent lyophilization of the pellet. Operating conditions of the extraction process such as pretreatment (NaOCl), temperature, ammonium laurate and biomass concentration and contact time were optimized. Best conditions for PHA extraction from MMC turned out to be: i) a pre-treatment with NaClO to break cell walls at 85 °C for 1 h of, followed by ii) a treatment with lauric acid in a lauric acid-to-biomass ratio of 2:1 for 3 h of contact time at pH-value 10. Notably, the pre-treatment with NaOCl resulted in slightly, but not dramatically higher purity and extraction yields than the sole use of surfactant (50).

Hypotonic disintegration of PHA-rich biomass

Haloarchaea, members of the extremely halophilic branch of the archaea domain, thrive best at extreme salinities in the range between 2 and 5 M NaCl. These ancient microorganisms show an extraordinarily high inner-osmotic pressure to balance the high medium osmolarity outside the cells (51). As for the first time described in 1990 by Rodriguez-Valera and Lillo for PHA-rich *Hfx. mediterranei* biomass, subjecting biomass of such extremely halophiles to hypotonic environments (i.e., distilled water) leads to rapid disrupture of cells without the use of organic extraction solvents. By this treatment, cells burst immediately, and PHA granules are set free. Due to the lower specific mass of PHA granules compared to the non-PHA cell matter, a two-phase system, consisting of a lower aqueous phase containing the cell debris, and a skimmed phase at the top mainly consisting of PHA granules, can conveniently be obtained by sedimentation and centrifugation. The two phases can be separated by simply removing the heavier, aqueous phase. Intact PHA granules obtained by this process can be further purified if required (52). This was shown later by Battacharyya and colleagues, who cultivated *Hfx. mediterranei* on vinasse as inexpensive carbon source. The accumulated PHBHV copolyester was released as granules by hypo-osmotic treatment, and further purified by application of aqueous NaOCl solution (53). This process was later enhanced by Alsafadi and Al-Mashaqbeh, who disrupted PHA-rich *Hfx. mediterranei* biomass by a combination of hypo-osmotic shock, SDS treatment and vortexing, which was followed by sodium hypochlorite treatment for generation of highly pure product (54).

In addition to the numerous reports for *Hfx. mediterranei*, a similar process was developed by Hezayen *et al.* for release of PHA granules by hypo-osmotic cell lysis of *Halopiger aswanensis* DSM 13151 biomass in distilled water and separation of granules by differential centrifugation (55). However, there are still various reports in the recent literature, which resort to PHA recovery from extremely halophilic biomass by halogenated solvents, mainly to get a uniform, ultra-pure product. This was shown by Salgaonkar and Bragança, who cultivated the haloarchaeon *Halogeometricum borinquense* on hydrolyzed sugar cane bagasse. Oven-dried *Hgm. borinquense* biomass was mortar-and-pestle ground, and Soxleth-extracted by chloroform (56).

Mechanical disintegration of PHA-rich biomass

Although doing without the use of organic solvents, mechanical techniques for biomass disintegration often do not deliver required product purity, and need additional purification of released PHA granules. In addition, such mechanical methods like, e.g., using bead mills, high pressure homogenization, vortexing, or ultrasonication, are often complicated for upscaling, are inefficient for biomass containing only low PHA loads, and are based on a cascade of subsequent process steps. As an example, disruption of PHA-rich cells of *C. necator* by high-pressure homogenization was successfully demonstrated by the German company ARGUS Umweltbiotechnologie, Berlin. Here, a high-density cultivation broth (about 200 g/L PHA-rich biomass) was almost quantitatively disintegrated by using two subsequent homogenization cycles at 800 kg/cm^2. The generated mixture of cellular material, *inter alia* PHA granules, can afterwards be separated by means of dissolved air floatation. The authors of this article underline that development of this process is still on the laboratory stage, and gets complicated in case of lipophilic residues in the cultivation broth (14).

Biological digestion of non-PHA biomass by animals

More recently, it was successfully demonstrated that the non-PHA fraction of biomass could selectively be digested by some animals, most prominently by the meal worm *Tenebrio molitor*, which results in excretion of PHA granules of astonishing purity and, at the same time, generates worms as a valuable animal protein resource, which, in consequence, could act as feed and even contribute to enhance food security in disadvantaged global regions. Although this method takes long time, it might become feasible if integrating PHA production in biorefinery concepts encompassing insect farming. The principles and potential of this process were comprehensively summarized by Chee and colleagues (57). The experiments underlying this review involved the feeding of meal worms with PHA-rich C. *necator* biomass, whereby the animals digested the non-PHA biomass material. The remaining white fecal pellets were simply washed with alkaline water. Purity of PHA obtained by this novel process reached 94%, which is sufficient for various applications of PHA in the non-medical field; moreover, the product did not show any reduction of molecular mass (58). It was also shown that the excreted granules obtained by digestion of lyophilized *C. necator* biomass retained their native spherical morphology. Comparing with chloroform extraction experiments using the same biomass, it was shown that this biological method does not cause reduction of molecular mass or increase of Đ, and simple post-treatment of the granules using water, SDS, slightly elevated temperature and strongly diluted HCl results in an almost 100% pure product (59). In addition to meal worms, even the digestion of lyophilized *C. necator* H16 cells containing about 40 wt.-% PHB as the sole diet source by rats (Sprague Dawley) was reported. The test animals readily excreted fecal pellets of whitish color, which contained about 82-97 wt.% PHB; molecular mass of the PHB granules recovered by this biological method had similar molecular mass compared to PHB extracted from the same biomass sample by the established chloroform method (60).

Genetic engineering to facilitate PHA recovery

Finally, recovery of PHA biopolyesters can be simplified by genetic engineering techniques. In this context, *Staphylococcus aureus* nuclease was successfully expressed by Rodríguez Gamero and colleagues in PHA producing species like *C. necator* and *Delftia acidovorans*. Nuclease expression and excretion into the cultivation medium reduces the viscosity of the mixture generated by biomass disruption via a "Constant Systems Cell Disrupter One Shot" apparatus caused by released nucleic acids (61).

Another genetic approach is the expression of the PHA synthesis genes in strains like *E. coli*, which are no natural PHA producers. Their cell wall is simply not suitable to maintain cell integrity when harboring excessive loads of inclusion bodies like PHA. Consequently, such high loads lead to the burst of cells even under mild biomass treatment after harvest. This was demonstrated by Choi and Lee, who equipped *E. coli* cells with *C. necator* PHA synthesis genes. The resulting recombinant strain accumulated more than 70 wt.-% PHA in biomass. By simply stirring the biomass with 0.2 N NaOH at 30°C for 1 h, PHA of a purity of more than 95% was recovered (62).

Comparative studies

In order to provide direct comparison of different PHA recovery methods individually tested in above discussed studies, Fernández-Dacosta and colleagues presented a techno-economic analysis for PHA recovery from MMC biomass. Three standard methods, namely simple alkaline (NaOH) treatment, the combined application of surfactant (SDS) and NaOCl, and solvent (DMC and ethanol) extraction. The comparison of obtained results showed that the alkaline treatment method had a lower ecological footprint, and slightly outperformed the NaOCl method also in economic terms. For this scenario, 70% of total production costs were allotted to downstream processing, while this value increased to 73% for NaOCl treatment. Authors explained this by the need for the surfactant SDS to support hypochlorite digestion of biomass. High energy de-

mands for distillative recovery of solvents (DMC and ethanol) resulted in the highest ecological impact and also highest cost shares (79% of total PHA production costs) among the three compared techniques (63). Comparing different solvent-based methods, Righi and colleagues calculated superior environmental performance for DMC in comparison with halogenated solvents, and underlined that PHA extraction from dried biomass outperforms extraction from wet bacterial sludge form the environmental perspective (64).

To summarize the different PHA recovery methods discussed in this review article, **Table 1** shows a compilation of time demand, chemicals requirement, product purity and yields, scalability, and applicability for diverse microbial PHA production strains.

Table 1. Comparison of different PHA isolation methods (update and extensive expansion of [14])

Recovery method	Method suitable for strains / types of PHA:	Time expenditure	Throughput of chemicals	Scalability to industrial scale?	Recovery yields	Product purity	Reduction of molar mass	Selected references
Organic solvents								
Chloroform extraction	All strains	Medium	High	No	High	High	Medium	[17]
Combined application of chloroform and aqueous NaOCl solution	All strains	Medium	High	No	High	High	Medium	[19]
"Agroferm method" based on cyclic carbonates)	All strains	Medium	Medium	Yes	Medium	High	High	[28]
Brazilian "PHB/ISA method" using fusel alcohols	All strains	High	Extraction solvents available in-house	Yes	High	Low/ Medium	High	[31]
Extraction with lactic acid esters	All strains	Medium/ High	High	Yes	Low/ Medium	Medium	High	[65]
Ethanol extraction	Mutant strains, *mcl*-PHA producers. For *scl*-PHA: in pressurized vessels	Medium	Low-Medium (easily recyclable solvent)	Yes	Low	Low/ Medium	High	[32]
GBL extraction	All strains	Medium	High	No	Low	Medium	Low	[23]
Cyclohexanone extraction	All strains	Medium	High	Yes	Medium	Medium	Low	[23]
Dimethyl carbonate (DMC)	All strains	Low	Medium	To be tested	Medium	High	Medium	[26, 35, 63, 64]
1,2-propylene carbonate	All strains	Low	Low-Medium (easily recyclable solvent)	Yes	Medium	High	Medium	
Acetone under reflux	Pseudomonads (*mcl*-PHA producers) Fractionation of low crystalline *scl*-PHA fractions	Medium	Medium (easily recyclable solvent)	No	High	High	Low	[18, 29] [22]
Acetone under elevated temperature and pressure	All strains	Low	Medium (easily recyclable solvent)	Adequate equipment to be developed; data only for lab-scale	High	High	Low	[22]

Table 1. *Continued*

Recovery method	Method suitable for strains / types of PHA:	Time expenditure	Throughput of chemicals	Scalability to industrial scale?	Recovery yields	Product purity	Reduction of molar mass	Selected references
Ethyl acetate	All strains	Medium	Medium (easily recyclable solvent)	To be tested	High	Medium	Medium-High	[24, 33]
Supercritical solvents								
Supercritical sCO_2	Tested only for restricted number of strains; *scl*-PHA and *mcl*-PHA	Medium	High (addition of modifiers needed)	No	Low	Low	Low	[37, 39, 40]
Ionic liquids								
$C2_{mim}][MeO(H)PO_2]$	All strains	Low	High	To be tested	Medium	Medium	Low	[43, 44]
Biocatalytic and biological digestion of non-PHA cell mass								
Digestion of non-PHA cell material by enzyme cocktails ("ICI process")	All strains	Low	High	Yes	High	Low	No	[45, 46]
Biological digestion of non-PHA biomass by *Tenebrio molitor* and excretion of PHA granules with feces	All non-toxic microbes	High	No	Doubtful	High	Medium	Low	[57-59]
Chemical digestion of non-PHA cell mass								
Alkaline (NaOH) digestion of non-PHA cell material	Rec. *E.coli*; wild type strains with high contents of PHA like *A. vinelandii*	Low	Low	Yes	Medium	High	Depends on incubation time and temperature	[62]
Combined SDS/ hypochlorite treatment	All strains	Medium	Medium	Yes	High	High	Negligible (depends on hypochlorite concentration)	[47, 63]
Hypochlorite digestion of non-PHA cell material	All strains	Medium	Medium	No	Medium	Medium	Medium-High	[48]
Switchable anionic surfactants (NH_4-laurate)	All strains	Medium	Medium	Yes	Medium	High	Low	[26, 50]
Mechanical and osmotic disrupture of cells								
Mechanical disintegration of PHA-rich cells (High-pressure homogenization, ultrasonication, vortexing, bead mills)	All strains (Ultrasonication for fragile strains like *Haloferax mediterranei* or rec. *E. coli*)	Low	No	Yes	Medium	Low	No	[14]
Cell disruption in hypotonic medium	Tested for highly osmophilic strains like *Haloferax mediterranei* or *Halopiger aswanensis*	Low	No	Yes	High	Medium	No	[52-55]

Conclusions

As shown in the presented case studies published during the last years, it is no trivial task to answer the question about the optimum PHA recovery process. Decisive factors for or against a method under consideration are:

- The production strain: Fragile or robust cell wall (wild-type or recombinant strain)? High inner-osmotic pressure? Mixed or pure microbial culture? Gram-positive or Gram-negative strain? Eukaryotes like yeasts or recombinant plants?
- The intracellular PHA mass fraction
- In-house availability of extraction solvents to reduce recovery costs
- Wet or dry biomass to be extracted? (Energy-demanding drying step needed?)
- What is the required product purity in dependence on expected application of the polymer
- What is the acceptable reduction of molecular mass or increase of Đ, respectively. Here, compromises have often to be made between a typical gain in product purity and recovery yield on the one hand, and a loss in molecular mass and higher Đ on the other hand.
- Is the method established only on research scale or already on pilot or industrial scale?
- Can safety regulations associated to a given recovery process (extraction in pressurized vessels, handling of halogenated and other irritating compounds on a larger scale, etc.) be obeyed?
- Is a strategy for recovery and/or recycling of chemicals available? This covers economic (re-use of compounds) and environmental (generation of harmful, e.g., halogenated or surfactant-rich, waste streams) aspects.

Consequently, every new PHA production process in development needs full consideration of diverse realistic downstream processing scenarios; this consideration has to become an integral part of a holistic technoeconomic and environmental assessment of the new process to be optimized. Most of all, it should be considered that production of "sustainable bioplastics", as it is the aim of the entire PHA technology, intrinsically can not resort to the application of extraction solvents, which are based on petrochemistry, which cannot be re-used, and which exert toxic effects on the environment and people in the industrial plant exposed to them. As shown, diverse alternative recovery techniques were already developed at least on the laboratory scale, and now need to pass the proof of concept for industrial implementation.

Conflict of interest statement

The author declares that there is not conflicts of interest.

References

1. Obruca S, Sedlacek P, Slaninova E, Fritz I, Daffert C, Meixner K, Sedrlova Z., Koller M. Novel unexpected functions of PHA granules. Appl Microbiol Biotechnol 2020; 104: 4795–4810.
2. Kourmentza C, Plácido J, Venetsaneas N, Burniol-Figols A, Varrone C, Gavala HN, Reis MAM. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 2017; 4(2): 55.
3. Koller M, Maršálek L, Miranda de Sousa Dias M, Braunegg G. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New Biotechnol. 2017; 37: 24-38.
4. Brigham CJ, Riedel SL. The potential of polyhydroxyalkanoate production from food wastes. Appl Food Biotechnol 2018; 6(1): 7-18.
5. Moradi M, Rashedi H, Mofradnia SR, Khosravi-Darani K, Ashouri R, Yazdian F. Polyhydroxybutyrate production from natural gas in a bubble column bioreactor: simulation using COMSOL. Bioengineering 2019; 6(3): 84.
6. Liu LY, Xie GJ, Xing DF, Liu BF, Ding J, Ren NQ. Biological conversion of methane to olyhydroxyalkanoate: Current advances, challenges, and perspectives. Env Sci Ecotechnol 2020; 100029.
7. Troschl C, Meixner, K, Drosg B. Cyanobacterial PHA production—Review of recent advances and a summary of three years' working experience running a pilot plant. Bioengineering 2017; 4(2): 26.
8. Karmann S, Panke S, Zinn M. Multiple nutrient-limited growth and polyhydroxybutyrate (PHB) production of Rhodospirillum rubrum on syngas is restricted by the availability of CO as energy source. Front Bioeng Biotechnol 2019; 7: 59.
9. Yu LP, Wu FQ, Chen GQ. Next-Generation Industrial Biotechnology-transforming the current industrial biotechnology into competitive processes. Biotechnology J. 2019; 14(9): 1800437.
10. Chen X, Yu L, Qiao G, Chen GQ. Reprogramming Halomonas for industrial production of chemicals. J Ind Microbiol Biotechnol 2018; 45(7): 545-554.
11. Sabapathy PC, Devaraj S, Meixner K, Anburajan P, Kathirvel P, Ravikumar Y., Zabed HM, Qi X. Recent developments in polyhydroxyalkanoates (PHAs) production in the past decade–A review. Bioresour Technol 2020; 123132.
12. Choi j, Lee SY. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol 1999; 51(1): 13-21.
13. Pérez-Rivero C, López-Gómez JP, Roy I. A sustainable approach for the downstream processing of bacterial polyhydroxyalkanoates: State-of-the-art and latest developments. Biochem Eng J 2019; 150: 107283.
14. Koller M, Niebelschütz H, Braunegg G. Strategies for recovery and purification of poly(l-3-hydroxyalkanoates) (PHA) biopolyesters from surrounding biomass. Eng Life Sci 2013; 13(6): 549-562.
15. Madkour MH, Heinrich D, Alghamdi MA, Shabbaj II, Steinbüchel A. PHA recovery from biomass. Biomacromolecules 2013; 14(9): 2963-2972.
16. Kunasundari B, Sudesh K. Isolation and recovery of microbial polyhydroxyalkanoates. Express Polym Lett 2011; 5(7): 620–634.
17. Ramsay JA, Berger E, Voyer R, Chavarie C, Ramsay BA. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol Tech 1994; 8(8): 589-594.
18. Rebocho AT, Pereira JR, Neves LA, Alves VD, Sevrin C, Grandfils C., Freitas F, Reis MAM. Preparation and characterization of films based on a natural P(3HB)/mcl-PHA blend obtained through the co-culture of *Cupriavidus necator* and *Pseudomonas citronellolis* in apple pulp waste. Bioengineering 2020; 7(2): 34.
19. Ojha N, Das N. Process optimization and characterization of polyhydroxyalkanoate copolymers produced by marine *Pichia kudriavzevii* VIT-NN02 using banana peels and chicken feather hydrolysate. Biocat Agri Biotechnol 2020; 101616.
20. Braunegg G, Bona R, Koller M. Sustainable polymer production. Polym Plast Technol Eng 2004; 43(6): 1779-1793.
21. Aramvash A, Moazzeni Zavareh F, Gholami Banadkuki N. Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*. Eng Life Sci 2018; 18(1): 20-28.
22. Koller M, Bona R, Chiellini E, Braunegg G. Extraction of short-chain-

length poly-(l-hydroxyalkanoates) (*scl*-PHA) by the "anti-solvent" acetone under elevated temperature and pressure, Biotechnol Lett 2013; 35(7): 1023-1028.
23. Jiang G, Johnston B, Townrow D, Radecka I, Koller M, Chaber P, Adamus G, Kowalczuk M. Biomass Extraction Using Non-Chlorinated Solvents for Biocompatibility Improvement of Polyhydroxyalkanoates. Polymers 2018; 10(7): 731.
24. Riedel SL, Brigham CJ, Budde CF, Bader J, Rha C, Stahl U, Sinskey AJ. Recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from *Ralstonia eutropha* cultures with non-halogenated solvents. Biotechnol Bioeng 2013; 110(2): 461-470.
25. Yang YH, Jeon JM, Kim JH, Seo HM, Rha C, Sinskey AJ, Brigham CJ. Application of a non-halogenated solvent, methyl ethyl ketone (MEK) for recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-*co*-HV)) from bacterial cells. Biotechnol Bioproc Eng 2015; 20(2): 291-297.
26. Samorì C, Basaglia M, Casella S, Favaro L, Galletti P, Giorgini L, Marchi D, Mazzocchetti L, Torri C, Tagliavini E. Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. Green Chem 2015; 17(2): 1047-1056.
27. Furrer PC. Medium-chain-length poly((*R*)-3-hydroxy-alkanoates): From biosynthesis towards medical applications, thesis (Doctoral dissertation) at Eidgenössische Technische Hochschule ETH Zürich, Nr. 17654, 2008
28. Lafferty RM, Heinzle E. (1979). U.S. Patent No. 4,140,741. Washington, DC: U.S. Patent and Trademark Office.
29. Cerrone F, Radivojevic J, Nikodinovic-Runic J, Walsh M, Kenny ST, Babu R, O'Connor KE. Novel sodium alkyl-1, 3-disulfates, anionic biosurfactants produced from microbial polyesters. Colloid Surface B 2019; 182: 110333.
30. Asrar J, Paster MD, Solodar AJ, Strausser FE, Kurdikar Devdatt. L (2000) Methods of PHA extraction and recovery using non-halogenated solvents. European Patent 90,975,788
31. Nonato R, Mantelatto P, Rossell C. Integrated production of biodegradable plastic, sugar and ethanol. Appl Microbiol Biotechnol 2001; 57(1-2): (2001) 1-5.
32. García A, Pérez D, Castro M, Urtuvia V, Castillo T, Díaz-Barrera A, Espín G, Pena C. Production and recovery of poly-3-hydroxybutyrate (P(3HB)) of ultra-high molecular weight using fed-batch cultures of *Azotobacter vinelandii* OPNA strain. J Chem Technol Biotechnol 2019; 94(6): 1853-1860.
33. Gahlawat G, Kumar Soni S. Study on sustainable recovery and extraction of Polyhydroxyalkanoates (PHAs) produced by *Cupriavidus necator* using waste glycerol for medical applications. Chem Biochem Eng Q 2019; 33(1): 99-110.
34. Gahlawat G, Soni SK. Valorization of waste glycerol for the production of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer by *Cupriavidus necator* and extraction in a sustainable manner. Bioresour Technol 2017; 243: 492-501.
35. de Souza Reis GA, Michels MH, Fajardo GL, Lamot I, de Best JH. Optimization of green extraction and purification of PHA produced by mixed microbial cultures from sludge. Water 2020; 12(4): 1185.
36. Reverchon E. Supercritical fluid extraction and fractionation of essential oils and related products. J Supercritical Fluids 1997; 10(1); 1-37.
37. Hampson JW, Ashby RD. Extraction of lipid-grown bacterial cells by supercritical fluid and organic solvent to obtain pure medium chain-length polyhydroxyalkanoates. J Am Oil Chem Soc 1999; 76(11): 1371-1374.
38. Williams JR, Clifford AA, Al-Saidi SH. Supercritical fluids and their applications in biotechnology and related areas. Mol Biotechnol 2002; 22(3): 263.
39. Khosravi-Darani K, Vasheghani-Farahani E, Shojaosadati SA, Yamini Y. Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for poly(*R*-hydroxybutyrate) recovery. Biotechnol Progr 2004; 20(6): 1757-1765.
40. Hejazi P, Vasheghani-Farahani E, Yamini Y. Supercritical fluid disruption of *Ralstonia eutropha* for poly(β-hydroxybutyrate) recovery. Biotechnol Progr 2003; 19(5): 1519-1523
41. Rogers RD, Seddon KR. Ionic liquids-solvents of the future? Science 2003; 302 (5646): 792-793
42. Tang S, Baker GA, Zhao H. Ether-and alcohol-functionalized task-specific ionic liquids: attractive properties and applications. Chem Soc Rev 2012; 41(10): 4030-4066.
43. Fujita K, Kobayashi D, Nakamura N, Ohno H. Direct dissolution of wet and saliferous marine microalgae by polar ionic liquids without heating. Enzyme Microb Tech 2013; 52(3): 199-202.
44. Kobayashi D, Fujita K, Nakamura N, Ohno H. A simple recovery process for biodegradable plastics accumulated in cyanobacteria treated with ionic liquids. Appl Microbiol Biotechnol 2015; 99(4): 1647-1653.
45. Holmes PA, Lim GB. Separation process. US Patent 4910145, 1990
46. Kachrimanidou V, Kopsahelis N, Vlysidis A, Papanikolaou S, Kookos IK, Martínez BM, ... & Koutinas AA. Downstream separation of poly (hydroxyalkanoates) using crude enzyme consortia produced via solid state fermentation integrated in a biorefinery concept. Food Bioprod Proc 2016; 100: 323-334.
47. Marudkla J, Patjawit A, Chuensangjun C, Sirisansaneeyakul S. Optimization of poly (3-hydroxybutyrate) extraction from *Cupriavidus necator* DSM 545 using sodium dodecyl sulfate and sodium hypochlorite. Agric Natural Res 2018; 52(3): 266-273
48. Berger E, Ramsay BA, Ramsay JA, Chavarie C. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol Tech 1989; 3: 227–232.
49. Mahansaria R, Bhowmik S, Dhara A, Saha A, Mandal MK, Ghosh R, Mukherjee J. Production enhancement of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in *Halogeometricum borinquense*, characterization of the bioplastic and desalination of the bioreactor effluent. Process Biochem. 2020; 94: 243-257.
50. Mannina G, Presti D, Montiel-Jarillo G, Suárez-Ojeda ME. Bioplastic recovery from wastewater: a new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures. Bioresour Technol 2019; 282: 361-369.
51. Koller M. Polyhydroxyalkanoate biosynthesis at the edge of water activitiy-haloarchaea as biopolyester factories. Bioengineering 2019; 6(2): 34.
52. Rodríguez-Valera F, Lillo JG. Halobacteria as producers of poly-ß-hydroxyalkanoates. In: Dawes EA (Ed.) Novel Biodegradable Microbial Polymers, NATO ASI Series (Series E: Applied Sciences), vol 186, Springer, Dordrecht, The Netherlands, pp. 425-426.
53. Bhattacharyya A, Pramanik A, Maji SK, Haldar S, Mukhopadhyay UK, Mukherjee J. Utilization of vinasse for production of poly-3-(hydroxybutyrate-*co*-hydroxyvalerate) by *Haloferax mediterranei*. AMB express 2012; 2(1): 34.
54. Alsafadi D, Al-Mashaqbeh O. A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-*co*-hydroxyvalerate) from olive mill wastewater by *Haloferax mediterranei*. New Biotechnol 2017; 34: 47-53.
55. Hezayen FF, Rehm BHA, Eberhardt R, Steinbüchel A. Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl Microbiol Biotechnol 2000; 54(3): 319-325.
56. Salgaonkar BB, Bragança JM. Utilization of sugarcane bagasse by *Halogeometricum borinquense* strain E3 for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). Bioengineering 2017; 4(2): 50.
57. Chee JY, Lakshmanan M, Jeepery IF, Hairudin NHM, Sudesh K.

The potential application of *Cupriavidus necator* as polyhydroxyalkanoates producer and single cell protein: a review on scientific, cultural and religious perspectives. Appl Food Biotechnol 2019; 6(1): 19-34.
58. Ong SY, Zainab-L I, Pyary S, Sudesh K. A novel biological recovery approach for PHA employing selective digestion of bacterial biomass in animals. Appl Microbiol Biotechnol 2018; 122(5): 2117–2127.
59. Murugan P, Han L, Gan CY, Maurer FH, Sudesh K. A new biological recovery approach for PHA using mealworm, *Tenebrio molitor*. J Biotechnol 2016; 239: 98-105.
60. Kunasundari B, Murugaiyah V, Kaur G, Maurer FH, Sudesh K. Revisiting the single cell protein application of *Cupriavidus necator* H16 and recovering bioplastic granules simultaneously. PloS One 2013; 8(10): e78528.
61. Rodríguez Gamero JE, Favaro L, Pizzocchero V, Lomolino G, Basaglia M, Casella S. Nuclease expression in efficient polyhydroxyalkanoates-producing bacteria could yield cost reduction during downstream processing. Bioresour Technol 261 (2018) 176-181.
62. Choi JI, Lee SY. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. Biotechnol Bioeng 62; 2000: 546-553.
63. Fernández-Dacosta C, Posada JA, Kleerebezem R, Cuellar MC, Ramirez A. Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: techno-economic analysis and *ex-ante* environmental assessment. Bioresour Technol 2015; 185: 368-377.
64. Righi S, Baioli F, Samorì C, Galletti P, Tagliavini E, Stramigioli C, Tugnoli A, Fantke P. A life cycle assessment of poly-hydroxybutyrate extraction from microbial biomass using dimethyl carbonate. J Clean Prod 2017; 168: 692-707.
65. Metzner K, Sela M, Schaffer J. Agents for extraction polyhydroxyalkane acids. European Patent EP0848759, 1998