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SPECIAL FEATURE: ORIGINAL ARTICLE

Recent Advances in Sludge Management

Comparative evaluation of polyhydroxyalkanoates recovery methods for a mixed microbial culture derived from waste activated sludge

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Abstract
The production of polyhydroxyalkanoates (PHAs) using a mixed microbial culture (MMC) derived from waste activated sludge (WAS) is a promising strategy for converting wastewater treatment plants into biorefinery facilities. Despite many studies on PHA production, the recovery of PHA stored in WAS-derived MMC has received less attention. In this study, we comparatively evaluated three solvent extraction, three cellular digestion, and two mechanical disruption methods to determine their effectiveness in the recovery of PHA from WAS-derived MMC. Chloroform extraction, sodium hydroxide digestion, and ultrasonic disruption were selected as effective candidates in initial screenings, and their processing conditions were optimized. While PHA recovery by the optimized chloroform extraction was 74%, the recovered polymers contained ignorable impurities and had superior molecular properties. In contrast, sodium hydroxide digestion and ultrasonic disruption enabled PHA recovery without loss, but the purity ($>73\%$ and $\geq 80\%$, respectively) and molecular properties of the recovered polymers required some improvement. Our findings suggest that these PHA recovery methods would be applicable in PHA production using WAS-derived MMC with pre- or post-processing as necessary, though they did not necessarily satisfy all the requirements in PHA production systems.

Keywords Waste activated sludge · Mixed microbial culture · Polyhydroxyalkanoate · Recovery · Downstream processing

Introduction

Wastewater treatment aims to remove contaminants in wastewater generated from human activities and has greatly contributed to protecting water quality, and thus human health and wildlife. However, for a sustainable and low-carbon society, wastewater treatment plants (WWTPs) are expected to contribute to the circular economy by evolving into wastewater biorefineries coupled with water resource recovery [1]. In particular, the continuous increase in the quantity of waste activated sludge (WAS) generation in WWTPs is a global problem because sludge treatment is cost- and energy-consuming and causes a large environmental burden (50% of operating cost and 40% of greenhouse gas emission in WWTPs [2]). In addition to minimizing WAS generation, a more intensive use of WAS for the recovery of resources, especially value-added materials, is desired in the context of a circular economy [2, 3].

Polyhydroxyalkanoates (PHAs) are promising value-added materials that can be produced using WAS [1, 4]. PHAs are a group of polyesters synthesized by many species of naturally occurring bacteria [5]. They are completely biodegradable and biocompatible thermoplastic materials with polymer characteristics similar to those of conventional petroleum-based plastics such as polypropylene [5]. Therefore, PHAs are promising alternative plastic materials applicable for various purposes [6]. Nonetheless, the high costs of pure culture-based PHA production limit PHA commercialization [7]. In contrast, PHA production using mixed microbial cultures (MMCs) as the biocatalyst has multiple merits, including simpler processes, cost-effectiveness, and lower energy consumption, since MMCs do not require sterile conditions and infrastructure for an axenic bioprocess and have a wider metabolic potential than single strains, which allows the use of organic waste streams as feedstock [7–9].

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Besides, PHA production using WAS as the biocatalyst can simultaneously reduce the expenses and energy needed for waste disposal, thus representing an opportunity to convert traditional end-of-pipe wastewater treatment into biorefineries [4, 8].

The processes involved in PHA production using MMCs basically consist of (i) an enrichment step, where PHA-accumulating bacteria in WAS are selectively enriched to prepare MMCs, (ii) the PHA accumulation step to maximize PHA storage in MMCs, and (iii) the PHA recovery step to obtain the biopolymer for industrial use [7, 10, 11]. Due to substantial efforts made during the last two decades, the biotechnological methodologies for producing PHA with high efficiency and yield using MMCs (steps (i) and (ii)) have been reasonably well-established. In some cases, a very high PHA accumulation comparable to that by pure cultures (ca. 90% (w/w)) was achieved using WAS-derived MMCs [12, 13]. However, the downstream processing step is a crucial part of the PHA production system because of its large impact on the final production quality and its significant contribution to the production costs (approximately 50%), and thus a bottleneck in the PHA value chain [14, 15].

In the recovery step, PHA is recovered from non-PHA cellular materials (NPCM), including polypeptides, phospholipids, nucleic acids, and peptidoglycan. A variety of PHA recovery methods, grouped into solvent extraction, cellular (i.e., NPCM) digestion, and mechanical disruption, have been studied and proven to be effective for pure cultures [1, 9, 16, 17]. The pure cultures usually have high PHA accumulation, increasing cellular fragility [18], making PHA recovery relatively easy. In contrast, MMCs often have lower PHA content and appear to be more robust because of their complex composition and the matrix of extracellular polymeric substances [9]. Thus, a method that is effective for recovering PHA from pure cultures is not necessarily effective for recovering PHA from MMCs. However, studies focusing on the recovery of PHA from MMCs are limited [19]. In particular, few studies have comparatively evaluated the effectiveness of different methods grouped into different categories (solvent extraction, cellular digestion, and mechanical disruption) on PHA recovery from MMCs [20]. In addition, only a few studies have comprehensively characterized different methods in light of the efficiency of PHA recovery and purity, as well as the polymer properties of the recovered PHA [19–21].

Therefore, we aimed to comparatively evaluate the effectiveness of the various methods of PHA recovery from WAS-derived MMCs. Eight methods, including three solvent extraction, three cellular digestion, and two mechanical disruption methods, were chosen as potential methods to be screened. All these selected methods have been proven high recovery yield and purity for PHA recovery from pure cultures. To our knowledge, no study has evaluated the PHA recovery abilities of multiple methods in all of the three categories. Furthermore, this study specially focused on the application of individual methods in a single run without repeated treatments or sequential processing with other methods to avoid complicated or laborious operations in practical applications. First, the eight methods were assessed for their ability to recover PHA from WAS-derived MMCs based on PHA recovery efficiency and purity, and the promising methods were selected in each category. Subsequently, PHA recovery was performed using the three selected methods under various processing conditions to identify optimal conditions required to maximize PHA recovery abilities. In addition, the molecular and thermal properties of PHA recovered by the three methods under the optimal conditions were investigated. This study provides useful knowledge for practical implementation of PHA production systems using WAS-derived MMCs in WWTPs.

Materials and methods

Preparation of MMC

A lab-scale sequencing batch reactor (SBR) with a working volume of 10 L was set up to enrich the PHA-accumulating bacteria from WAS through the aerobic dynamic feeding method. Basal salt medium (BSM) [22] containing sodium acetate (324 mg C/L) was used as the influent. The SBR cycle was fixed at 12 h, comprising an initial feeding phase (15 min), a reaction phase (645 min), a sedimentation phase (30 min), and an effluent withdrawal phase (30 min). The SBR operation was initiated by inoculating WAS collected from a municipal WWTP (Osaka, Japan) at a mixed liquor suspended solids (MLSS) concentration of 5 g/L into 10 L medium. During the reaction phase, air was supplied using an XP-40 air pump (Techno Takatsuki, Osaka, Japan) through an air stone to keep the dissolved oxygen concentration above 1.0 mg/L. Based on previous knowledge of efficient enrichment of PHA-accumulating bacteria, the SBR temperature was maintained at 22 ± 1 °C, and the pH was not controlled [23]. The SBR operation was continued for nearly 1 month (43 to 60 cycles) to obtain a stable feast-to-famine ratio, and the resultant MMC was applied for PHA accumulation.

PHA accumulation in MMC

PHA accumulation in the established MMC was conducted with a fed-batch cultivation mode [24]. The MMC was washed twice with nitrogen- and phosphorus-deficient BSM, and inoculated at an MLSS concentration of 2 g/L in a 2 L beaker containing 1.6 L of nitrogen- and phosphorus-deficient BSM supplemented with 1080 mg-C/L of sodium

acetate. The culture was incubated at 22 ± 1 °C, stirred at 400 rpm using an RS-6DN magnetic stirrer (AS ONE, Osaka, Japan), and aerated at 2.0 L/min using an XP-40 air pump. During the cultivation, the pH in the culture increased with the consumption of acetate. Therefore, 1.5 M acetic acid solution was additionally supplied using a TDP-51 pH controller (Toko Chemical Laboratories, Tokyo, Japan) to maintain the pH at 8.0 ± 0.1. After a 48-h cultivation, the MMC in which PHA was accumulated at 41.0–50.9% (w/w) was centrifuged (2500 × *g*, 10 min) and lyophilized using an FDU-1000 freeze dryer (EYELA, Tokyo, Japan) before use in PHA recovery experiments.

Screening of effective PHA recovery methods

The candidates selected to screen effective PHA recovery methods are: (i) extraction with chloroform, dichloromethane and dimethyl carbonate, (ii) digestion with sodium hydroxide (NaOH), sodium hypochlorite (NaClO) and sodium dodecyl sulfate (SDS), and (iii) mechanical disruption by ultrasonication and bead milling. These methods have been applied to recover PHA from pure cultures and MMCs with relatively high recovery yields and purities [9, 11, 17, 19, 25, 26]. Aliquots (100 mg) of the lyophilized sludge were put into a 10 mL glass tube, or 15 mL centrifuge tube. PHA recovery was conducted using each method under the standard effective conditions reported below.

For the three solvent extraction methods, 4 mL of each solvent was added to the lyophilized sludge. Extraction was performed with the following conditions: chloroform extraction at 58 °C for 30 min [27], dichloromethane extraction at 50 °C for 4 h [18], and dimethyl carbonate extraction at 90 °C for 1 h [18]. After chloroform extraction, 4 mL ultrapure water was added, the tube was shaken vigorously for 5 min, and the organic layer was recovered. Meanwhile, the samples obtained after dichloromethane or dimethyl carbonate extraction were centrifuged (2500 × *g*, 10 min), and the supernatant was filtered through a 0.45 µm polytetrafluoroethylene membrane filter. Finally, the polymer was recovered by evaporating the solvents overnight at 60 °C.

In the cellular digestion, the lyophilized sludge was suspended in 10 mL of 0.2 M NaOH or NaClO (5% Cl_2) or 6 mL of SDS (3%(w/v)) and reacted with shaking (180 rpm) under the following conditions: at 30 °C for 1 h for NaOH treatment [21], at 100 °C for 1 h for NaClO treatment [18], and at 90 °C for 3 h for SDS treatment [18]. Mechanical cell disruption through ultrasonication was performed in 10 mL ultra pure water using a VCX-130 PB ultrasonic processor (Sonics & Materials, Inc., CT, USA) with an amplitude of 80% for 2 min [25]. For bead milling, the lyophilized sludge was dispersed in 5 mL of McIlvaine buffer (pH 2.6), and a microorganism lysing VK01/VK05 kit (Bertin Technologies, Montigny-le-Bretonneux, France) was used for cell disruption by vortexing for 2 min at maximum strength. After each treatment, the samples were centrifuged, and the recovered pellets were washed twice with ultra pure water and dried at 60 °C overnight.

Optimization of processing conditions for selected methods

The optimum processing conditions were determined for chloroform extraction, NaOH digestion, and ultrasonic disruption that were selected in the first screening. For chloroform extraction, the effects of sludge loading (20–150 mg per 4 mL chloroform), temperature (20–100 °C), and reaction time (5–90 min) were assessed. For NaOH digestion, the effects of sludge loading (20–1500 mg per 10 mL NaOH solution), NaOH concentration (2–1000 mM), temperature (10–70 °C), and reaction time (1–120 min) were assessed. For ultrasonic disruption, the effects of sludge loading (208–150 mg per 10 mL of solvent), pH (2–12), and reaction time (0.5–15 min) were tested. Ultra pure water (pH 7) and Britton-Robinson buffer (pH 2, 5, 9, and 12) were used as the solvents for ultrasonic cell disruption. The lyophilized sludge was prepared, and post-treatment of the recovered pellets was conducted, as mentioned above.

Quantification of PHA amount

The recovered sludge pellets after treatment with each PHA recovery method were centrifuged (2500×*g*, 10 min) when necessary and lyophilized. The lyophilized samples were pretreated by methanolytic decomposition [28]. In addition, the lyophilized sludge samples before PHA recovery treatment were subjected to a chloroform extraction-methanolytic decomposition method to quantify the PHA accumulated in the MMCs [22, 28]. PHA contents were determined using a GC2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an Inert Cap WAX-HT capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; GL Science, Tokyo, Japan) [22, 28]. Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (Sigma-Aldrich, MO, USA) was used as the authentic standard, and the PHA contents were determined as the sum of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV).

Characterization of PHA

Gel permeation chromatography (GPC) was performed to determine the weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (M_w/M_n; PDI) using a GPC-104 (Shoko Scientific, Kanagawa, Japan) equipped with Shodex GPC LF-404 × 2 column (Showa Denko, Kanagawa, Japan). For GPC analysis, the recovered PHA samples were dissolved in hexafluoroisopropanol (HFIP) containing 10 mM sodium trifluoroacetate at 0.2% (w/v) and filtered

through a 0.2-μm membrane filter. HFIP containing 10 mM sodium trifluoroacetate was used as the eluent at a flow rate of 0.3 mL/min at 40 °C. A differential scanning calorimetry (DSC) analysis was performed using a DSC 8500 (PerkinElmer Japan, Kanagawa, Japan) to determine the thermal properties (glass transition temperature (T_g) and melting temperature (T_m)) of the recovered PHA samples. The DSC analysis was conducted under a nitrogen atmosphere (flow rate: 50 mL/min). The temperature program used was a first heating from −30 to 190 °C at 10 °C/min and a second heating from −30 to 200 °C at 10 °C/min after rapid cooling. Both analyses were conducted at the DJK Corporation (Kanagawa, Japan).

Calculations

PHA recovery performance was evaluated based on the PHA recovery efficiency and purity [29]. These values were calculated using the following equations:

$$PHA\ recovery\ efficiency\ (\%) = 100 \times \frac{Mass\ of\ PHA\ in\ the\ recovered\ pellet}{Mass\ of\ PHA\ in\ the\ lyophilized\ sludge}$$

$$PHA\ purity\ (\%) = 100 \times \frac{Mass\ of\ PHA\ in\ the\ recovered\ pellet}{Mass\ of\ the\ recovered\ pellet}$$

PHA recovery and purity were compared between the different treatment methods and conditions using Duncan's multiple range test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using R software (v3.4.3).

Results and discussion

Selection of effective PHA recovery methods

The performances of three solvent extraction methods (chloroform, dichloromethane, and dimethyl carbonate), three cellular digestion methods (NaOH, NaClO, and SDS), and two mechanical disruption methods (ultrasonication and bead milling) in recovering PHA from lyophilized MMC sludge was examined under standard conditions to select a promising method within each category. Upon the selection, PHA recovery efficiency was given the highest priority, followed by PHA purity, as maximizing the amount of PHA that can be recovered is important. In addition, when multiple methods within a category had comparable PHA recovery performance, a method with milder treatment conditions and less environmental impact was considered favorable.

Of the three solvents, chloroform exhibited the highest PHA recovery performance in terms of recovery efficiency (71.1%) and purity (95.9%) (Fig. 1a). A PHA recovery efficiency of 39.4% was obtained on extraction with dichloromethane, and this was lower than that obtained from MMC by the same method in previous study (recovery: 55.9% [21]). However, largely different PHA recovery performance (15–86%) was reported for dichloromethane extraction, even for pure cultures [30, 31]. This evidence suggests that the PHA extraction ability of dichloromethane may vary notably depending on the microbial species; thus, dichloromethane extraction may not be suitable for stable PHA recovery from MMCs consisting of a variety of microorganisms. Dimethyl carbonate reportedly exhibits excellent performance in PHA recovery from pure cultures, with PHA recovery efficiency

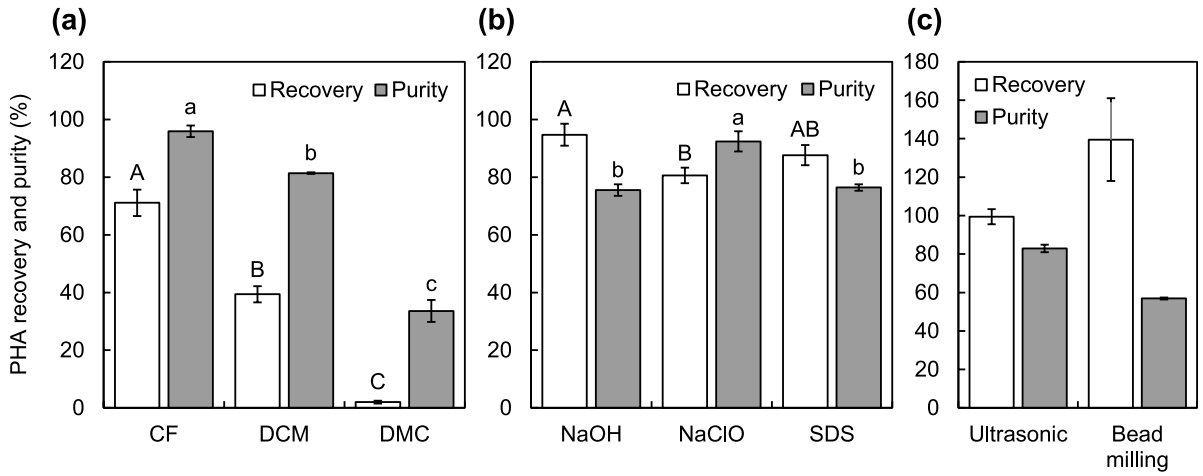

Fig. 1 PHA recovery and purity obtained by various methods within the categories of (**a**) solvent extraction, (**b**) cellular digestion, and (**c**) mechanical cell disruption. *CF* chloroform, *DCM* dichloromethane, *DMC* dimethyl carbonate, *NaOH* sodium hydroxide, *NaClO* sodium hypochlorite, *SDS* sodium dodecyl sulfate. Different uppercase and lowercase letters indicate a significant difference between the means for recovery and purity data, respectively (Duncan's multiple range test, $p < 0.05$)

and purity of 94% and 93%, respectively [14]. However, the PHA recovery performance in this study was very low regarding both recovery and purity (Fig. 1a). Similarly, low PHA recovery from MMCs using dimethyl carbonate (4–49%) was reported in previous studies [18, 32], indicating that dimethyl carbonate is unsuitable for PHA recovery from MMCs.

Notably, all three cellular digestion methods had superior PHA recovery performances, with > 80% recovery efficiency and 75% purity (Fig. 1b). The highest PHA recovery efficiency (94.7%) and purity (92.4%) were obtained using NaOH and NaClO digestions, respectively. The PHA recovery abilities of the three methods were comparable to those found in previous PHA recoveries from MMCs: 80–100% recovery and 54–100% purity with NaOH digestion [20, 21, 33], 50–100% recovery and 86–99% purity with NaClO digestion [20, 29, 33], and 67–94% recovery and 42–93% purity with SDS digestion [18, 21, 29]. This evidence confirmed the stable PHA recovery abilities of all methods, irrespective of the microbial composition of the MMC. Contrary to the similarly high performance, the three methods differed in terms of treatment temperature and duration: 30 °C and 1 h in NaOH treatment, 100 °C and 1 h in NaClO treatment, and 90 °C and 3 h in SDS treatment. Therefore, NaOH treatment, which could achieve similar performance with less energy input, appeared to be more promising than the other treatments considering the environmental impact.

Of the two mechanical disruption methods, ultrasonication exhibited a superior PHA recovery ability, with 99.4% recovery and 82.9% purity (Fig. 1c). In contrast, in bead milling, PHA recovery efficiency exceeded 100% and purity was 56.2%. The over 100% recovery percentage with low purity in bead milling was likely attributable to the inclusion of fine beads in the recovered PHA samples, which poses a risk of causing problems in practical use. Therefore, ultrasonication was proven to be more practically useful.

Based on the above results, chloroform extraction, NaOH cell lysis, and ultrasonic cell disruption methods were chosen for further studies to optimize the treatment conditions.

Optimization of chloroform extraction

Solvent extraction using halogenated solvents, such as chloroform, destroys the cell wall and proceeds with the release and solubilization of PHA [17]. Chloroform extraction is one of the most commonly used and proven effective methods for PHA recovery from pure cultures and MMCs [9]. The effects of sludge loading, temperature, and treatment time on the recovery efficiency and purity of PHA from an MMC sludge were examined as potential parameters affecting the PHA recovery performance of chloroform extraction. These parameters are important in practical use because reducing the solvent amount, treatment temperature, and treatment time can save the costs and energy for extraction as well as mitigate potential degradation of the recovered biopolymer [29, 34]. The standard conditions were set as follows: sludge loading of 100 mg (per 4 mL chloroform), temperature at 58 °C, and reaction time of 30 min.

The recovery efficiency was stable at 70–74% at a sludge loading of 20–100 mg but significantly declined at 150 mg (Fig. 2a). An excellent purity (> 97%) of the recovered biopolymer was maintained, irrespective of the sludge loading. Efficient PHA recovery with minimum solvent use is preferable in practical PHA production. Thus, the optimum sludge loading was determined to be 100 mg per 4 mL of chloroform. PHA recovery through chloroform extraction at 20–100 °C revealed that the recovery efficiency improved linearly from 20–58 °C and was unchanged at higher temperatures (Fig. 2b). A previous study performing the recovery of PHA stored in *Alcaligenes eutrophus* found higher PHA recovery in chloroform extraction at 61 °C than at 25 °C [30]. The purity of the recovered polymer was also superior at 58 °C compared to 20–40 °C, reaching 100%. Thus, 58 °C was considered the optimal temperature for chloroform extraction, achieving the highest recovery and complete purity of PHA with the lowest energy input for heating. The treatment time also had a significant influence (Fig. 2c). Prolonging the treatment duration from 5 to 30 min improved recovery efficiency and purity. However, further extension did not elevate the PHA recovery performance but rather impaired the recovery efficiency, suggesting that 30 min was the appropriate duration for chloroform extraction.

Chloroform extraction with a sludge loading of 100 mg per 4 mL at 58 °C for 30 min, which was the best condition determined in this study, achieved a PHA recovery efficiency and purity of 74.1% and 100%, respectively. This PHA recovery ability was comparable to that obtained by chloroform extraction from pure cultures (recovery: 55–69%, purity: 92–97%) [27, 30] and that obtained in the recovery of PHA from lyophilized MMC biomass by chloroform extraction followed by ethanol precipitation (recovery: 81.7%, purity: 95.1%) [20]. Our findings and those of previous studies suggest that chloroform extraction is especially useful for PHA recovery without impurities, and its PHA recovery performance is unaffected by the co-presence of diverse microorganisms and complicated NPCM in MMCs. The use of halogenated solvents such as chloroform is hampered by their potentially harmful impact on health and the environment [35]; however, potential risks can be mitigated by recovering and recycling the solvent [36], which may also be beneficial to the operating cost.

Optimization of NaOH digestion

Strong alkaline solutions, such as NaOH, can hydrolyze and saponify cellular components such as proteins and

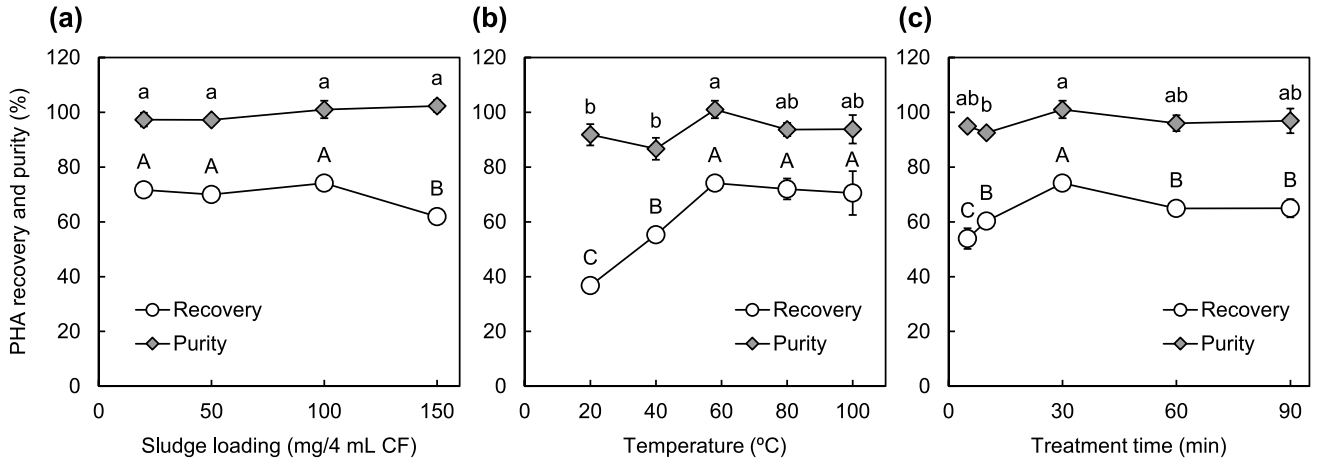

Fig. 2 PHA recovery and purity obtained by chloroform extraction with different conditions of (**a**) sludge loading, (**b**) temperature, and (**c**) treatment time. Standard conditions were sludge loading of 100 mg per 4 mL chloroform, temperature at 58 °C, and treatment time of 30 min. Different uppercase and lowercase letters indicate a significant difference between the means for recovery and purity data, respectively (Duncan's multiple range test, $p < 0.05$)

lipopolysaccharides, making the microbial cell membrane partially permeable and enabling the separation and purification of PHA inside bacterial cells [37]. Previous studies have reported that the alkaline dosage and the reaction temperature and time can positively or negatively affect the recovery and purity of the polymer in PHA extraction by alkaline digestion [19, 38–41]. The effects of sludge loading and the above three parameters on PHA recovery and purity from MMC were examined under the following standard conditions: treatment at 30 °C for 30 min with 100 mg sludge loading in 10 mL of 200 mM NaOH solution.

Notably, PHA recovery efficiency and purity were maximized at a sludge loading of 200 mg and declined with further loading (Fig. 3a). The decline at sludge loadings of ≥ 500 mg was likely caused by insufficient NPCM saponification and digestion. PHA recovery and purity were also improved with increase in NaOH concentration from 2 to 100 mM, reaching 100% recovery and 73.2% purity at 100 mM (Fig. 3b). However, 1000 mM NaOH caused a significant decline in PHA recovery. The increase in the performance of cell lysis and PHA recovery with increase in NaOH concentration has also been found in pure cultures [42] and MMCs [19, 21]. Jiang et al. [21] conducted PHA recovery from an MMC sludge without lyophilization using NaOH treatment at 20–1000 mM and reported that PHA recovery was the highest (98.0%) at 100 mM, and the purity increased gradually with increase in NaOH concentration within the test range. The reduced recovery by an excessive NaOH dose was likely attributable to the degradation of the PHA granules released into the medium [19]. NaOH digestion experiments at 10–70 °C showed that complete PHA recovery was achieved at 10–30 °C, and further increment of the treatment temperature lowered the recovery efficiency (Fig. 3c). In addition, no significant difference was observed in the purity of PHA recovered at 10–30 °C. Thus, it was suggested that room temperature without external heating was sufficient for efficient PHA recovery by NaOH treatment. When the duration of the NaOH treatment was extended from 1 to 120 min, no statistically significant difference was observed in the PHA recovery performance, although the maximum values of the mean PHA recovery efficiency and purity was obtained after 5 min of treatment (Fig. 3d). In the saponification/digestion process, NaOH is a reactant and its concentration declines as cell lysis progresses [19]. Consequently, a striking digestion effect in the NaOH treatment is exhibited during the initial short period, and thereafter the effect plateaus. Our results suggested that only a few minutes is sufficient for efficient cell disintegration and PHA recovery following NaOH treatment. These results are consistent with previous findings on PHB recovery from pure culture, which demonstrated no improved or rather negative effects on PHB recovery and purity by NaOH treatment at higher temperatures and for prolonged durations [43]. In this study, over 100% recovery efficiencies were obtained under several conditions of NaOH treatment (Fig. 3). These results were likely attributed to the application of a chloroform extraction-methanolytic decomposition method, which is a conventional standard method but cannot completely recover PHA, in the determination of the PHA accumulated in the MMC sludge before the recovery treatment experiments.

Thus, the optimal conditions for NaOH treatment were determined as follows: sludge loading of 200 mg per 10 mL of 100 mM NaOH solution and treatment at room

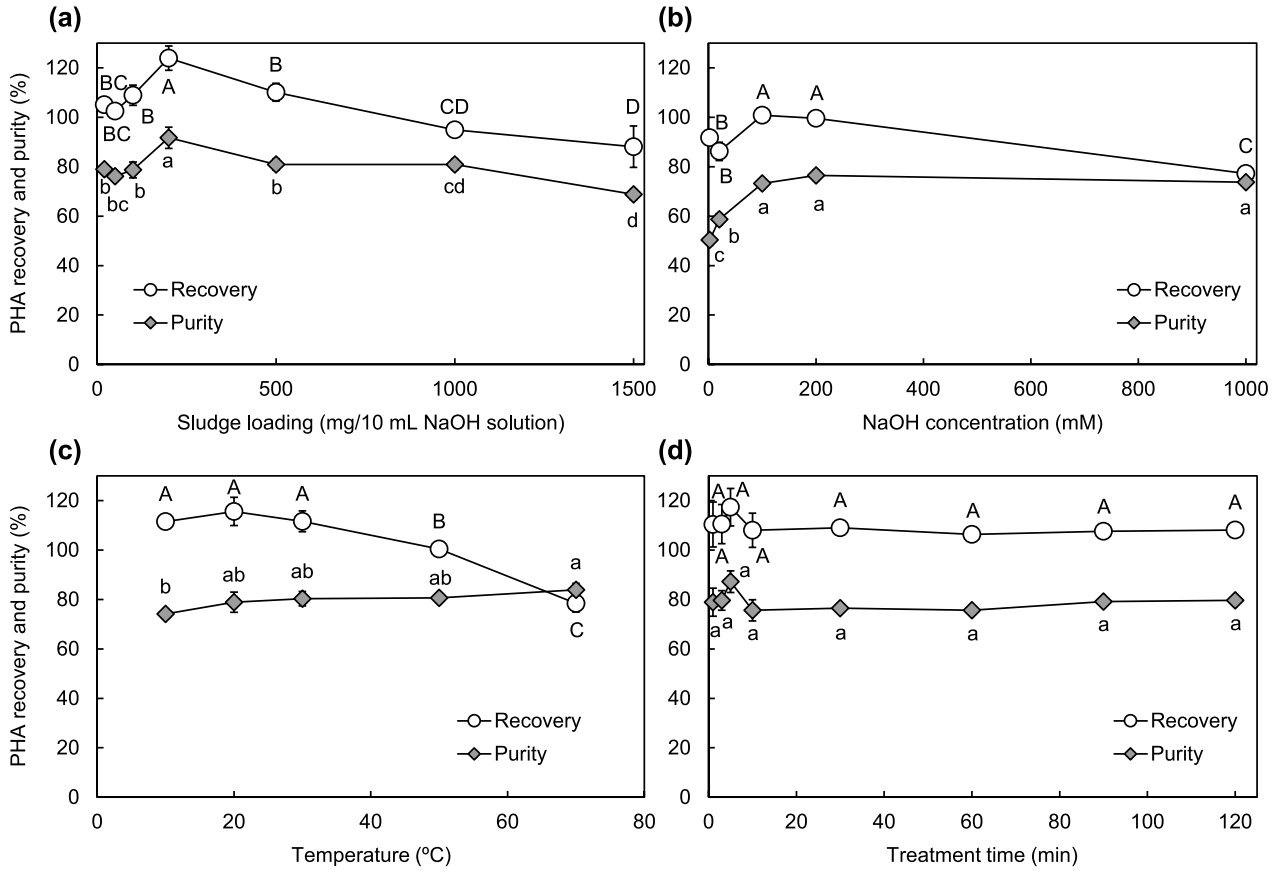

Fig. 3 PHA recovery and purity obtained by NaOH digestion with different conditions of (**a**) sludge loading, (**b**) NaOH concentration, (**c**) temperature, and (**d**) treatment time. Standard conditions were sludge loading of 100 mg per 10 mL solution, NaOH concentration of 200 mM, temperature at 30 °C, and treatment time of 30 min. Different uppercase and lowercase letters indicate a significant difference between the means for recovery and purity data, respectively (Duncan's multiple range test, $p < 0.05$)

temperature (10–30 °C) for 5 min. Through the optimized conditions, NaOH treatment was suggested to completely recover PHA from MMC with a high purity (> 73%). The nearly complete recovery of PHA by NaOH digestion was also observed in other trials using pure cultures [42] and MMC [21]. Thus, NaOH digestion is a useful method for the complete recovery of PHA. However, the purity of the recovered PHA needs to be improved. For example, combining NaOH extraction with other treatments, such as SDS lysis [21], and adding a pretreatment with NaCl [44], are plausible strategies for improving the purity of the biopolymer.

Optimization of ultrasonic disruption

Ultrasonication involves using sound waves to disrupt the microbial cell wall and disintegrate sludge flocs, leading to the release of cellular substances into the aqueous solution. It has often been applied as a pretreatment or support for other methods to improve PHA recovery from pure cultures [25, 45–47] and MMCs [18]. We examined the effects of sludge loading, pH, and treatment time on the PHA recovery performance of ultrasonic disruption. The standard conditions were set at 100 mg sludge per 10 mL of solvent, 7 (neutral), and 2 min, respectively.

When PHA recovery was performed using ultrasonication with the sludge loading of 20–150 mg, the highest PHA recovery efficiency and purity were obtained at 50–100 mg (Fig. 4a). No significant difference was observed in performance between the two sludge loadings. However, a sludge loading of 150 mg significantly lowered the PHA recovery performance. This could be because the high sludge concentration prevented ultrasonication, leading to insufficient cell disintegration. Thus, the optimum sludge loading was determined to be 100 mg per 10 mL of solvent. Notably, PHA recovery using ultrasonication was almost complete, irrespective of the pH condition (Fig. 4b). The recovery efficiency slightly declined under extremely acidic and alkaline conditions; however, no significant difference was observed in the recovery efficiency at pH 7–9 and pH 2 or 12. In contrast, the purity of the recovered PHA was positively correlated with pH, and an alkaline pH facilitated PHA recovery from MMCs with high purity. This superior performance

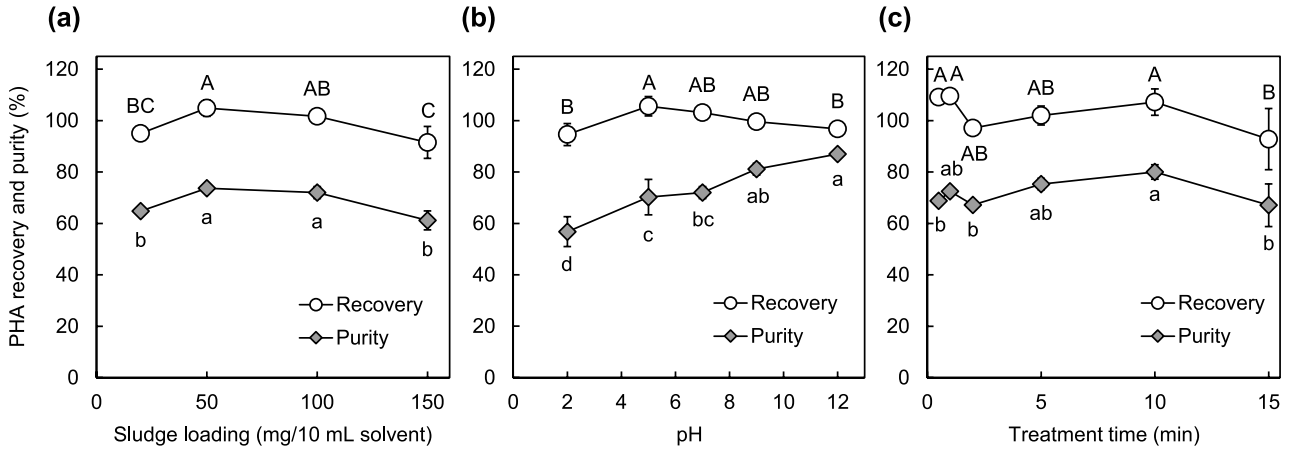

Fig. 4 PHA recovery and purity obtained by ultrasonic disruption with different conditions of (**a**) sludge loading, (**b**) pH, and (**c**) treatment time. Standard conditions were sludge loading of 100 mg per 10 mL solution, pH 7, and treatment time of 2 min. Different uppercase and lowercase letters indicate a significant difference between the means for recovery and purity data, respectively (Duncan's multiple range test, $p < 0.05$)

under alkaline conditions is attributed to the synergistic effects of ultrasonic disruption, and alkaline digestion and saponification [19]. Furthermore, while nearly complete recovery was achieved with up to 10 min of ultrasonic treatment, extending the treatment duration to 15 min notably decreased the recovery efficiency (Fig. 4c). In contrast, the purity of the recovered PHA was improved at a treatment duration of 2 to 10 min, reaching a maximum purity of 80%. However, it declined significantly with further prolongation. Thus, it is likely that the cellular components of the MMC sludge could not be sufficiently disintegrated within a short treatment duration (up to 5 min), resulting in low purity of the recovered PHA. In contrast, the PHA released from the cells appeared to be partly decomposed during long-term ultrasonication (15 min), leading to a decrease in the recovery efficiency.

Collectively, treatment for 10 min with a sludge loading of 100 mg per 10 mL of solvent at a pH of 12 was determined to be optimal for ultrasonic disruption. Under these conditions, ultrasonication resulted in a nearly complete recovery of PHA from MMCs with a purity of ≥ 80%. This PHA recovery performance was comparable to or superior to that from pure cultures [46, 47] and MMCs [19] obtained by other ultrasonication-assisted treatments.

Properties of PHA recovered by the optimized methods

The molecular and thermal properties of the recovered PHA are important parameters that affect their final applications, and they vary depending on the recovery strategy [44]. The molecular weight (M_w and M_n), PDI (M_w/M_n), and thermal properties (T_g and T_m) of the PHA recovered by the three methods under the optimized conditions were determined. The results are listed in Table 1, along with the properties of PHA recovered from MMCs in previous studies.

The M_w of the PHA samples recovered with the three methods in this study was 2.3–4.6 × 10^5 Da, with the largest M_w for chloroform extraction. All the M_w in this study were within the M_w range of PHA samples reported in the literature (1.0 × 10^4–4.0 × 10^6 Da) [29, 36], and appeared to be comparable to those of the commercial 3HB homopolymer [19, 48]. In the recovery of the 3HB homopolymer from *Cupriavidus necator* cultures, chloroform extraction resulted in a higher M_w compared to other methods (e.g., NaOH digestion) [43]. The PDI values (3.9–7.3) were also within the reported range but seemed relatively high compared to those of the commercial 3HB homopolymer [19, 48]. The noticeably greater variance in ultrasonication was likely resulted from the intense collisions between PHA and NPCM. Therefore, although all three optimized methods are applicable for PHA recovery with sufficient molecular weight, the relatively wide molecular weight distribution may leave room for improvement.

The T_m values of the PHA samples in this study (170–172 °C) were almost the same, irrespective of the recovery method, as found in a previous study that recovered the 3HB homopolymer in *Cupriavidus necator* cultures using different methods [43]. The T_m values in this study fell within the known T_m range of PHA (131–180 °C), and were similar to those of the 3HB homopolymer [27], but were higher than those produced in other MMC studies [20, 33, 48]. In addition, the T_g values of our PHA samples were similar to those of PHA samples produced by other MMCs [20, 33]. Given the evidence that the T_m of the copolymer of 3HB and 3HV decreases with increase in 3HV content [49], the relatively

Table 1 Molecular and thermal properties of PHA recovered from MMCs with chloroform extraction, NaOH digestion, and ultrasonic disruption under the optimal conditions in this study and with various methods reported in previous studies

Recovery method	PHA content in MMC (%, w/w)[a]	M_w ($\times 10^5$ Da)	M_n ($\times 10^5$ Da)	PDI	T_g (°C)	T_m (°C)	Reference
Chloroform	45.3 (3.1)	4.6	1.1	4.1	–1	172	This study
NaOH	45.3 (3.1)	2.3	0.59	3.9	–2	170	This study
Ultrasonic	45.3 (3.1)	3.2	0.44	7.3	–4	171	This study
NaClO	46 (11)	3.4–5.4	na	4–10	0	153	[33]
NaClO + ammonium laurate	52 (ca. 4)	0.918	0.418	2.2	na	na	[29]
NaClO + ammonium laurate	54 (47)	1.438	0.444	3.2	na	na	[29]
NaOH	70 (31)	2.45	1.33	1.84	–1.57	109.6	[20]
Chloroform	70 (31)	2.63	1.31	2.00	0.51	109.1	[20]
Chloroform	52 (na)	3.75	1.59	2.35	–17.3	136	[48]
Chloroform	70 (na)	4.62	2.08	2.22	–15.2	136	[48]
Ultrasonic + NaOH + NaClO	55–60 (na)	1.59–3.44	0.72–1.61	1.85–2.32	na	na	[19]

MMC mixed microbial culture, M_n number average molecular weight, M_w weight average molecular weight, *na* not available, *PDI* polydispersity index (= M_w/M_n), *PHA* polyhydroxyalkanoate, T_g glass transition temperature, T_m melting temperature

[a]The PHA are copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), specifically poly(3HB-*co*-3HV), and the values in parentheses indicate the percentage (%, w/w) of 3HV in the total PHA

higher T_m values of the PHA samples in this study resulted from their low 3HV contents (Table 1).

Conclusions

The effectiveness of the three solvent extraction, three cellular digestion, and two mechanical disruption methods for PHA recovery from WAS-derived MMCs were comparatively evaluated and characterized in terms of PHA recovery efficiency, and the purity and polymer properties of the recovered PHA. To our knowledge, this is the first study to comparatively evaluate and characterize the PHA recovery abilities of multiple methods in all three categories (i.e., solvent extraction, cellular digestion, and mechanical disruption) in their individual use without repetitive treatment and to identify the treatment conditions that maximize recovery performance. The major findings of this study are summarized as follows.

(1) Chloroform extraction, NaOH digestion, and ultrasonic disruption were the potential effective methods within the categories of solvent extraction, cellular digestion, and mechanical disruption methods, respectively.
(2) Chloroform extraction under optimum processing conditions could not recover all PHA in the MMC. The recovered polymers contained minimal impurities and maintained a high molecular weight with smaller variants, which are the most important features of PHA for its subsequent industrial use.
(3) NaOH digestion and ultrasonic disruption under the optimum conditions were proven effective in recovering PHA stored in the MMC without significant loss, which is another important point in PHA production using WAS-derived MMCs, considering the low PHA content in MMCs compared to industrial pure cultures. However, the polymers recovered by NaOH disruption and ultrasonic disruption contained certain impurities and had slightly lower molecular weights than those recovered by chloroform extraction, which requires further improvement.

The selected and optimized three PHA recovery methods could not necessarily satisfy all requirements for PHA production systems using WAS-derived MMCs. However, still three methods are considered useful for PHA recovery and that their effectiveness can be further enhanced by pre- or post-processing to compensate for their shortcomings. Meanwhile, even if MMCs were established with the same enrichment procedures, taxonomic groups and abundance of major PHA-accumulating bacteria in the MMCs differ, which leads to the difference in PHA accumulation [50] and consequently may affect the PHA recovery performance by each method. Therefore, further studies on MMCs with distinct microbial community compositions and PHA contents are needed to assess the versatility and variability of the selected methods and to explore the important factors in MMCs affecting the effectiveness of each method.

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Data availability Data will be made available on request.

Declarations

Competing Interest The authors declare that they have no conflict of interest.

References

1. Mannina G, Presti D, Montiel-Jarillo G, Carrera J, Suárez-Ojeda ME (2020) Recovery of polyhydroxyalkanoates (PHAs) from wastewater: a review. Bioresour Technol 297:122478. https://doi.org/10.1016/j.biortech.2019.122478
2. Gherghel A, Teodosiu C, De Gisi S (2019) A review on wastewater sludge valorisation and its challenges in the context of circular economy. J Clean Prod 228:244–263. https://doi.org/10.1016/j.jclepro.2019.04.240
3. Shi S, Xu G, Yu H, Zhang Z (2018) Strategies of valorization of sludge from wastewater treatment. J Chem Technol Biotechnol 93:936–944. https://doi.org/10.1002/jctb.5548
4. Arcos-Hernández M, Montaño-Herrera L, Murugan Janarthanan O, Quadri L, Anterrieu S, Hjort M, Alexandersson T, Karlsson A, Karabegovic L, Magnusson P, Johansson P, Bengtsson S, Morgan-Sagastume F, de Vegt O, Laycock B, Pratt S, Halley P, Lant P, Werker A (2015) Value-added bioplastics from services of wastewater treatment. Water Pract Technol 10:546–555. https://doi.org/10.2166/wpt.2015.063
5. Reddy CSK, Ghai R, Rashmi, Kalia VC (2003) Polyhydroxyalkanoates: an overview. Bioresour Technol 87:137–146. https://doi.org/10.1016/S0960-8524(02)00212-2
6. El-malek FA, Khairy H, Farag A, Omar S (2020) The sustainability of microbial bioplastics, production and applications. Int J Biol Macromol 157:319–328. https://doi.org/10.1016/j.ijbiomac.2020.04.076
7. De Donno Novelli L, Sayavedra SM, Rene ER (2021) Polyhydroxyalkanoate (PHA) production via resource recovery from industrial waste stream: a review of techniques and perspectives. Bioresour Technol 331:124985. https://doi.org/10.1016/j.biortech.2021.124985
8. Valentino F, Morgan-Sagastume F, Campanari S, Villano M, Werker A, Majone M (2017) Carbon recovery from wastewater through bioconversion into biodegradable polymers. N Biotechnol 37:9–23. https://doi.org/10.1016/j.nbt.2016.05.007
9. Pagliano G, Galletti P, Samorì C, Zaghini A, Torri C (2021) Recovery of polyhydroxyalkanoates from single and mixed microbial cultures: a review. Front Bioeng Biotechnol 9:624021. https://doi.org/10.3389/fbioe.2021.624021
10. Serafim LS, Lemos PC, Albuquerque MGE, Reis MAM (2008) Strategies for PHA production by mixed cultures and renewable waste materials. Appl Microbiol Biotechnol 81:615–628. https://doi.org/10.1007/s00253-008-1757-y
11. Kourmentza C, Plácido J, Venetsaneas N, Burniol-Figols A, Varrone C, Gavala HN, Reis MAM (2017) Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 4:55. https://doi.org/10.3390/bioengineering4020055
12. Johnson K, Jiang Y, Kleerebezem R, Muyzer G, van Loosdrecht MCM (2009) Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromol 10:670–676. https://doi.org/10.1021/bm8013796
13. Jiang Y, Marang L, Kleerebezem R, Muyzer G, van Loosdrecht MCM (2011) Polyhydroxybutyrate production from lactate using a mixed microbial culture. Biotechnol Bioeng 108:2022–2035. https://doi.org/10.1002/bit.23148
14. Samorì C, Basaglia M, Casella S, Favaro L, Galletti P, Giorgini L, Marchi D, Mazzocchetti L, Torri C, Tagliavini E (2015) Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. Green Chem 17:1047–1056. https://doi.org/10.1039/c4gc01821d
15. Saavedra del Oso M, Mauricio-Iglesias M, Hospido A (2021) Evaluation and optimization of the environmental performance of PHA downstream processing. Chem Eng J 412:127687. https://doi.org/10.1016/j.cej.2020.127687
16. Pérez-Rivero C, López-Gómez JP, Roy I (2019) A sustainable approach for the downstream processing of bacterial polyhydroxyalkanoates: state-of-the-art and latest developments. Biochem Eng J 150:107283. https://doi.org/10.1016/j.bej.2019.107283
17. Kurian NS, Das B (2021) Comparative analysis of various extraction processes based on economy, eco-friendly, purity and recovery of polyhydroxyalkanoate: a review. Int J Biol Macromol 183:1881–1890. https://doi.org/10.1016/j.ijbiomac.2021.06.007
18. Samorì C, Abbondanzi F, Galletti P, Giorgini L, Mazzocchetti L, Torri C, Tagliavini E (2015) Extraction of polyhydroxyalkanoates from mixed microbial cultures: impact on polymer quality and recovery. Bioresour Technol 189:195–202. https://doi.org/10.1016/j.biortech.2015.03.062
19. Zou Y, Yang M, Tao Q, Zhu K, Liu X, Wan C, Harder MK, Liang B, Ntaikou I, Antonopoulou G, Lyberatos G, Zhang Y (2023) Recovery of polyhydroxyalkanoates (PHAs) polymers from a mixed microbial culture through combined ultrasonic disruption and alkaline digestion. J Environ Manage 326:116786. https://doi.org/10.1016/j.jenvman.2022.116786
20. Rodrigues AM, Franca RDG, Dionísio M, Sevrin C, Grandfils C, Reis MAM, Lourenço ND (2022) Polyhydroxyalkanoates from a mixed microbial culture: extraction optimization and polymer characterization. Polymers 14:2155. https://doi.org/10.3390/polym14112155
21. Jiang Y, Mikova G, Kleerebezem R, van der Wielen LAM, Cuellar MC (2015) Feasibility study of an alkaline-based chemical treatment for the purification of polyhydroxybutyrate produced by a mixed enriched culture. AMB Express 5:5. https://doi.org/10.1186/s13568-015-0096-5
22. Inoue D, Fukuyama A, Ren Y, Ike M (2019) Rapid enrichment of polyhydroxyalkanoate-accumulating bacteria by the aerobic dynamic discharge process: enrichment effectiveness, polyhydroxyalkanoate accumulation ability, and bacterial community

characteristics in comparison with the aerobic dynamic feeding process. Bioresour Technol Rep 7:100276. https://doi.org/10.1016/j.biteb.2019.100276
23. Inoue D, Suzuki Y, Sawada K, Sei K (2018) Polyhydroxyalkanoate accumulation ability and associated microbial community in activated sludge-derived acetate-fed microbial cultures enriched under different temperature and pH conditions. J Biosci Bioeng 125:339–345. https://doi.org/10.1016/j.jbiosc.2017.09.008
24. Inoue D, Fukuyama A, Ren Y, Ike M (2021) Optimization of aerobic dynamic discharge process for very rapid enrichment of polyhydroxyalkanoates-accumulating bacteria from activated sludge. Bioresour Technol 336:125314. https://doi.org/10.1016/j.biortech.2021.125314
25. Gutt B, Kehl K, Ren Q, Boesel LF (2016) Using ANOVA models to compare and optimize extraction protocols of P3HBHV from *Cupriavidus necator*. Ind Eng Chem Res 55:10355–10365. https://doi.org/10.1021/acs.iecr.6b02694
26. Macagnan KL, Alves MI, Moreira AdS (2019) Approaches for enhancing extraction of bacterial polyhydroxyalkanoates for industrial applications. In: Kalia V (ed) Biotechnological applications of polyhydroxyalkanoates. Springer, Singapore, pp 389–408. https://doi.org/10.1007/978-981-13-3759-8_15
27. Macagnan KL, Rodrigues AA, Alves MI, Furlan L, Kesserlingh SM, Moura AB, de Oliveira PD, McBride AJA, Moreira AS, Vendruscolo CT (2017) Simplified recovery process of *Ralstonia solanacearum*-synthesized polyhydroxyalkanoates via chemical extraction complemented by liquid-liquid phase separation. Quím Nova 40:125–130. https://doi.org/10.21577/0100-4042.20160162
28. Sakai K, Miyake S, Iwama K, Inoue D, Soda S, Ike M (2015) Polyhydroxyalkanoate (PHA) accumulation potential and PHA-accumulating microbial communities in various activated sludge processes of municipal wastewater treatment plants. J Appl Microbiol 118:255–266. https://doi.org/10.1111/jam.12683
29. Mannina G, Presti D, Montiel-Jarillo G, Suárez-Ojeda ME (2019) Bioplastic recovery from wastewater: a new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures. Bioresour Technol 282:361–369. https://doi.org/10.1016/j.biortech.2019.03.037
30. Ramsay JA, Berger E, Voyer R, Chavarie C, Ramsay BA (1994) Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol Tech 8:589–594. https://doi.org/10.1007/BF00152152
31. Furrer P, Panke S, Zinn M (2007) Efficient recovery of low endotoxin medium-chain-length poly([*R*]-3-hydroxyalkanoate) from bacterial biomass. J Microbiol Methods 69:206–213. https://doi.org/10.1016/j.mimet.2007.01.002
32. Montiel-Jarillo G, Morales-Urrea DA, Contreras EM, López-Córdoba A, Gómez-Pachón EY, Carrera J, Suárez-Ojeda ME (2022) Improvement of the polyhydrpxyalkanoates recovery from mixed microbial cultures using sodium hypochlorite pre-treatment coupled with solvent extraction. Polymers 14:3938. https://doi.org/10.3390/polym14193938
33. Villano M, Valentino F, Barbetta A, Martino L, Scandola M, Majone M (2014) Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. N Biotechnol 31:289–296. https://doi.org/10.1016/j.nbt.2013.08.001
34. Xiong B, Fang Q, Wei T, Wang Z, Shen R, Cheng M, Zhou W (2023) Chemical digestion method to promote activated sludge cell wall breaking and optimize the polyhydroxyalkanoate (PHA) extraction process. Int J Biol Macromol 240:124369. https://doi.org/10.1016/j.ijbiomac.2023.124369
35. Kosseva MR, Rusbandi E (2018) Trends in biomanufacture of polyhydroxyalkanoates with focus on downstream processing. Int J Biol Macromol 107:762–778. https://doi.org/10.1016/j.ijbiomac.2017.09.054
36. Nayır TY, Konuk S, Kara S (2023) Extraction of polyhydroxyalkanoate from activated sludge using supercritical carbon dioxide process and biopolymer characterization. J Biotechnol 364:50–57. https://doi.org/10.1016/j.jbiotec.2023.01.011
37. Anis SNS, Iqbal NM, Kumar S, Al-Ashraf A (2013) Increased recovery and improved purity of PHA from recombinant *Cupriavidus necator*. Bioengineered 4:115–118. https://doi.org/10.4161/bioe.22350
38. Anis SNS, Nurhezreen MI, Sudesh K, Amirul AA (2012) Enhanced recovery and purification of P(3HB-co-3HHx) from recombinant *Cupriavidus necator* using alkaline digestion method. Appl Biochem Biotechnol 167:524–535. https://doi.org/10.1007/s12010-012-9677-9
39. Mohammadi M, Hassan MA, Phang LY, Shirai Y, Man HC, Ariffin H (2012) Intracellular polyhydroxyalkanoates recovery by cleaner halogen-free methods towards zero emission in the palm oil mill. J Clean Prod 37:353–360. https://doi.org/10.1016/j.jclepro.2012.07.038
40. Mohammadi M, Hassan MA, Phang LY, Shirai Y, Man HC, Ariffin H, Amirul AA, Syairah SN (2012) Efficient polyhydroxyalkanoate recovery from recombinant *Cupriavidus necator* by using low concentration of NaOH. Environ Eng Sci 29:783–789. https://doi.org/10.1089/ees.2011.0255
41. Yu J, Plackett D, Chen LXL (2005) Kinetics and mechanism of the monomeric products from abiotic hydrolysis of poly[(*R*)-3-hydroxybutyrate] under acidic and alkaline conditions. Polym Degrad Stab 89:289–299. https://doi.org/10.1016/j.polymdegradstab.2004.12.026
42. Mohammadi M, Hassan MA, Shirai Y, Man HC, Ariffin H, Yee LN, Mumtaz T, Chong ML, Phang LY (2012) Separation and purification of polyhydroxyalkanoates from newly isolated *Comamonas* sp. EB172 by simple digestion with sodium hydroxide. Sep Sci Technol 47:534–541. https://doi.org/10.1080/01496395.2011.615788
43. López-Abelairas M, García-Torreiro M, Lú-Chau T, Lema JM, Steinbüchel A (2015) Comparison of several methods for the separation of poly(3-hydroxybutyrate) from *Cupriavidus necator* H16 cultures. Biochem Eng J 93:250–259. https://doi.org/10.1016/j.bej.2014.10.018
44. Anis SNS, Iqbal NM, Kumar S, Amirul AA (2013) Effect of different recovery strategies of P(3HB-*co*-3HHx) copolymer from *Cupriavidus necator* recombinant harboring the PHA synthase of *Chromobacterium* sp. USM2. Sep Purif Technol 102:111–117. https://doi.org/10.1016/j.seppur.2012.09.036
45. Arikawa H, Sato S, Fujiki T, Matsumoto K (2017) Simple and rapid method for isolation and quantitation of polyhydroxyalkanoate by SDS-sonication treatment. J Biosci Bioeng 124:250–254. https://doi.org/10.1016/j.jbiosc.2017.03.003
46. Ishak KA, Annuar MSM, Heidelberg T, Gumel AM (2016) Ultrasound-assisted rapid extraction of bacterial intracellular medium-chain-length poly(3-hydroxyalkanoates) (mcl-PHAs) in medium mixture of solvent/marginal non-solvent. Arab J Sci Eng 41:33–44. https://doi.org/10.1007/s13369-015-1833-4
47. Murugesan S, Iyyaswami R (2017) Low frequency sonic waves assisted cloud point extraction of polyhydroxyalkanoate from *Cupriavidus necator*. J Chromatogr B 1060:207–214. https://doi.org/10.1016/j.jchromb.2017.06.009
48. Zhou W, Colpa DI, Geurkink B, Euverink GW, Krooneman J (2022) The impact of carbon to nitrogen ratios and pH on the microbial prevalence and polyhydroxybutyrate production levels using a mixed microbial starter culture. Sci Total Environ 811:152341. https://doi.org/10.1016/j.scitotenv.2021.152341
49. Wang Y, Chen R, Cai J, Liu Z, Zheng Y, Wang H, Li Q, He N (2013) Biosynthesis and thermal properties of PHBV produced from levulinic acid by *Ralstonia eutropha*. PLoS ONE 8:e60318. https://doi.org/10.1371/journal.pone.0060318

50. Ren Y, Nakaho S, Inoue D, Ike M (2024) Polyhydroxyalkanotes-accumulating bacteria by aerobic dynamic discharge process to waste activated sludge from various wastewater treatment plants. Jpn J Water Treat Biol 60:25–37. https://doi.org/10.2521/jswtb.60.25