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Supercritical carbon dioxide-based approach for the recovery and purification of polyhydroxyalkanoates from mixed microbial cultures: A green approach for bioplastics production

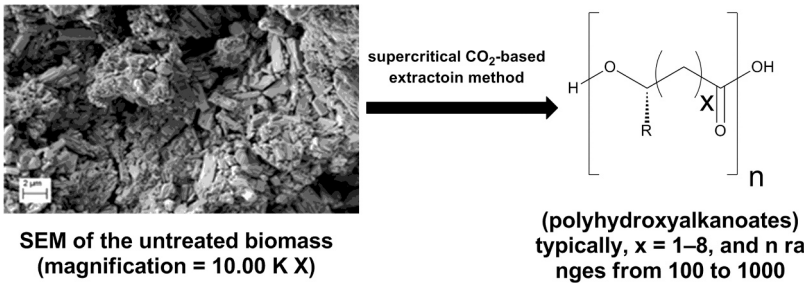
Farid Hajareh Haghighi [a], Gaia Salvatori [a], Sara Alfano [a], Laura Lorini [a], Francesco Valentino [b], Marianna Villano [a,c], Laura Chronopoulou [a,c,*], Cleofe Palocci [a,c,*]

[a] Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome 00185, Italy
[b] Department of Environmental Science, Informatics, and Statistics, Ca' Foscari, University of Venice, Via Torino 155, Venezia-Mestre 30172, Italy
[c] Research Center for Applied Sciences to the Safeguard of Environment and Cultural Heritage (CIABC), Sapienza University of Rome, Piazzale Aldo Moro 5, Rome 00185, Italy

HIGHLIGHTS

- Supercritical CO2- extraction selectively recovers PHAs from mixed microbial cultures.
- The use of co-solvents has a positive effect on PHAs extraction yields.
- PHAs extraction can be carried out at high ScCO2 pressure values.
- Biomass pretreatment with enzymes and H_2O_2 increases PHAs extraction yields.

GRAPHICAL ABSTRACT



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ABSTRACT

The widespread use of petroleum-based plastics has significant environmental consequences, including greenhouse gas emissions and long-term contamination of marine and terrestrial habitats. In contrast, bioplastics are a class of biopolymers, widely considered as the main alternative to conventional non-degradable plastics. Polyhydroxyalkanoates (PHAs) are a class of biodegradable polymers that have the potential to replace conventional polymers thanks to their similar properties. PHAs are currently produced using fermentation technologies, which require post-extraction purification procedures as well as the use of organic solvents for the removal of residual fermentation media to obtain high-quality products (e.g., medical devices). In the present study, supercritical CO_2-extraction ($scCO_2$) was employed as a green technology to selectively recover and purify PHAs from mixed microbial cultures using different multi-step protocols, including: the use of co-solvents (methanol, water and phosphate buffer solution) inside the $scCO_2$ cell, pre- and post-treatment of the biomass (with enzymes and H_2O_2), employing different times (2 h to 4 h), temperatures (35 °C to 40 °C) and pressures (20 MPa to 35 MPa) for the $scCO_2$-treatment. Among the tested protocols, the post-treatment of biomass with H_2O_2 and trypsin ($scCO_2$-H_2O_2-trypsin) resulted in the highest PHA purity and recovery with 97.2 % and 97.3 %, respectively. The results demonstrate the importance of $scCO_2$-based techniques as a valid alternative to conventional methods

* Corresponding authors at: Department of Chemistry, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome 00185, Italy.
E-mail addresses: laura.chronopoulou@uniroma1.it (L. Chronopoulou), cleofe.palocci@uniroma1.it (C. Palocci).

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that use chlorinated organic solvents. This highlights the importance of green technologies as a turning point in the industrial production of biopolymers starting from a complex biomass feedstock.

1. Introduction

Plastics are inexpensive synthetic or semi-synthetic polymers possessing unique properties such as lightness and durability, which play an important role in a wide range of applications in modern life [1,2]. In Europe, plastics are mainly used in goods (~10 %), automotive (~10 %), textiles (~15 %), construction (~20 %) and packaging (~40 %) [3]. However, about 99 % of them are produced from non-renewable resources such as petroleum/natural gas and charcoal, contributing to 20 % of the overall petroleum consumption [4,5]. Based on the reports by the Organisation for Economic Cooperation and Development (OECD), the global annual plastic production will reach 1.2 billion tons by 2060 [6]. Moreover, overuse of plastics should be considered in worldwide pandemics, for instance, the COVID-19 period led to the overproduction and overuse of single-use plastics, mainly as personal protective equipment, which accounts for a 10-fold increase in medical plastic waste [7,8]. Annually, millions of tons of non-degradable plastics accumulate in the environment. Biodegradable and bio-based biopolymers have been extensively studied as promising alternatives. During the last decades, different kinds of biopolymers such as polyhydroxyalkanoates (PHAs), aliphatic polyesters, polylactides and polysaccharides have been studied as alternatives. Among them, PHAs possess interesting biodegradable features, physical properties, slow hydrolytic degradation and biocompatibility, making them suitable for packaging and medical applications [9,10]. Together with polylactic acid and polybutylene succinate, PHAs are considered as green polymers of the future and are expected to gradually replace conventional plastics, since they have physico-chemical, thermal and mechanical properties similar to those of polypropylene (PP) and low-density polyethylene (LDPE) [11,12].

In general, PHA and PHA-based bioplastics can be used in various sectors including: 1) biodegradable and composite films for packaging [13,14]; 2) production of durable consumer goods [15,16]; 3) groundwater remediation as a source for slow carbon release [17,18]. PHAs are completely biodegradable thermoplastics that can be produced biologically by various microorganisms (e.g., *Bacillus, Rhodococcus, Pseudomonas* bacteria, etc.) through the fermentation of sugars or lipids for their intracellular storage of energy and carbon, [19,20]. PHA synthesis is stimulated by unbalanced growth of the microorganism during the fermentation and accumulation of PHA granules, as part of a survival mechanism of the microbes [21,22]. The composition of PHAs depends on both the type of bacteria from which they are synthesized and the feeedstock [23,24]. Among PHAs, polyhydroxybutyrate (PHB) has properties like polypropylene (PP) with good resistance to humidity. PHB is highly brittle and rigid while PHB copolymers including other fatty acids such as β-hydroxyvalerate acid (HV) are more ductile [25,26]. Despite the great PHA environmental advantages, their diffusion on the market is limited because of the poor sustainability of the recovery and purification stages [27,28]. In fact, the most effective recovery methods involve the use of chlorinated organic solvents and/or inorganic reagents (e.g. chloroform and sodium hypochlorite) for PHA granules solubilization, non-polymeric cellular matrix (NPCM) digestion or cell breakage [29,30].

Under the principles of green chemistry, processes should prevent waste production and reduce the use of solvents and hazardous chemicals to become safer and more energy efficient [31,32]. Following these guidelines, research has focused on developing the potential of greener processes and solvents such as the use of microwaves [33,34], ultrasounds [35,36], green solvents [37,38] and enzymatic pretreatments [39,40]. Supercritical carbon dioxide ($scCO_2$) has gained attention as a green solvent for the recovery and purification of PHAs from biomass [41], including crops and mixed microbial cultures [42,43]. This approach offers several advantages. First, selective extraction using supercritical CO_2 ($scCO_2$) enables efficient dissolution and separation of polyhydroxyalkanoates (PHAs) from biomass without relying on toxic solvents [44,45]. Second, it operates under mild conditions, with moderate temperatures and pressures that help reduce thermal degradation of PHAs [46,47]. Third, the method ensures enhanced purity, as $scCO_2$ extraction minimizes contamination from non-PHA cellular components, thereby improving the overall polymer quality [48,49]. Finally, it promotes sustainability, since CO_2 is both abundant and recyclable, making this technique well-aligned with environmentally friendly

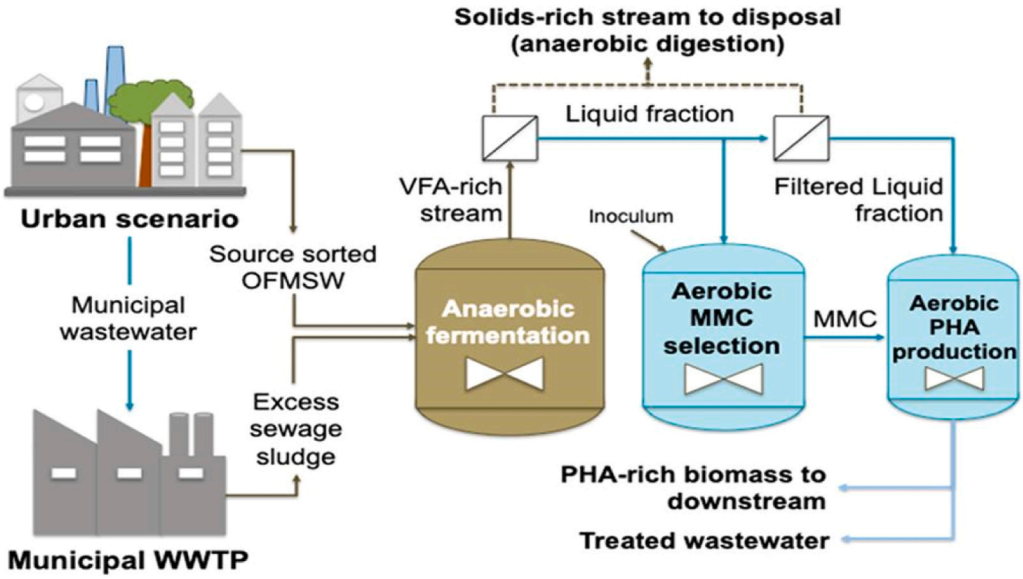

Fig. 1. Process diagram of the pilot plant (Reprinted from Ref. [64], Copyright © 2019 American Chemical Society). Abbreviation: WWTP (wastewater treatment plant); OFMSW (organic fraction of municipal solid waste); MMA (mixed microbial culture).

bioplastic production [50–53].

Enzymatic approaches for PHA recovery and purification are also gaining attention as sustainable alternatives to chemical extraction. These methods focus on breaking down non-PHA cellular components while preserving polymer integrity [54]. Key enzymatic strategies for PHA recovery involve several targeted approaches. One involves the use of cell lysis enzymes—including lysozyme, proteases, and lipases—which effectively degrade microbial cell walls and release PHAs without the need for harsh chemical treatments [55,56]. Another strategy is selective digestion, where enzymatic hydrolysis specifically targets non-PHA cellular materials, thereby enhancing polymer purity while minimizing degradation [57,58]. Additionally, these methods support biocompatible processing, offering a more environmentally friendly alternative to traditional solvent-based extraction techniques [59,60].

Regardless of the type of process chosen for recovery, pre-treatment is often used for biomass, such as freeze-drying, exposure to heat or use of alkali, which induces a denaturation of proteins and the weakening of the cell wall, facilitating subsequent extraction of the polymer [61–63].

The present work, with a view to the management and treatment of wastewater and municipal solid waste, exploits the possibility of using such waste products as feedstock for feeding microbial cells for intracellular PHA accumulation. Specifically, in this study, innovative green techniques were developed for the separation and purification of polyhydroxyalkanoates (PHA) from mixed microbial biomass. These methods employed either single-step or multi-step separation processes, incorporating at least one of the following environmentally conscious approaches: treatment of biomass with supercritical carbon dioxide ($scCO_2$), enzymatic hydrolysis using hydrolytic enzymes, or oxidation through hydrogen peroxide (H_2O_2). Each step was designed to enhance efficiency while minimizing environmental impact.

2. Materials and methods

2.1. Materials

Ultrapure water (18.3 MΩ·cm) was produced with a Zeneer Power I Scholar-UV (Full Tech Instruments, Rome, Italy) water purification system equipped with a 0.01 μm filter and used within 24 h. All chemicals, enzymes and organic solvents, analytical or HPLC grade, were supplied by Sigma (Italy) and used as received. All solvents, including liquid $CO_{2(l)}$ were supplied by Air Liquid (Rome, Italy).

2.2. Preparation of microbial biomass

The PHA-enriched biomass used in this work derived from the accumulation stage of a pilot plant MMC-PHA (MMA: mixed microbial culture) production process located in Treviso (at ATS S.p.A., Alto Trevigiano Servizi, Treviso, Italy), according to a procedure described in the literature [64]. The feedstock of the process consisted of a mixture of the organic fraction of municipal solid waste (OFMSW) and biological sludge. Fig. 1 shows the process diagram of the pilot plant: the anaerobic stage included the acidogenic fermentation of the organic feedstock in batch mode in a 380 L reactor's volume (reaction time of 6 days); the aerobic stages of culture selection of PHA- producing microorganisms and PHA accumulation were conducted in a Sequencing Batch Reactor (SBR, 120 L), HRT equal to the SRT of 1 day and a cycle time of 6 h, and a batch reactor (volume of 70–90 L inoculated with the selected biomass coming from the SBR), respectively. Both aerobic stages were fed with the fermentation stream rich into volatile fatty acids produced by the anaerobic stage. At the end of the accumulation step, the biomass enriched in PHA was thermally treated to stabilize the stored polymer content. Thermal stabilization consisted of an overnight heat treatment at 60 °C with a preceding heat shock at 145 °C for 30 min.

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Then, PHA was extracted from the PHA-enriched biomass (PHBV purity ca.51 % wt/wt, HV content ca. 20 % wt/wt) following a standard purification protocol in sodium hypochlorite: the PHA-rich dry biomass was suspended in a NaClO solution (1.5 % active Cl_2), then the suspension was kept under magnetic stirring overnight, centrifuged at 8500 rpm for 20 min and subsequently washed 3 times with distilled water. The wet pellet was dried in an oven at 60 °C.

The polymer fraction in the biomass, the purity of the extracted PHA and its composition were obtained by the gas chromatographic standard method (GC), described elsewhere [65]. The initial values of PHBV and HV content of the original biomass precursor was evaluated using a standard purification protocol in sodium hypochlorite [66]. In this standard protocol, the last step is to dry the pellet in the oven at 60 °C, before the GC analysis.

2.3. Pre-treatments on biomass: grinding and homogenization

The first pre-treatment was carried out on the granule sample by grinding the solid mass using a ceramic pestle and mortar to reduce the size of the solid fraction of the fermented product, allowing an improvement in the performance of the subsequent processes. To make homogenization more efficient, the pulverized sample was washed with different solvents: H_2O; H_2O/EtOH (in a 1:1 v/v ratio); *Candida rugosa* lipase (in 5 % H_2O_2 solution). The optimized protocol involved the treatment of 1 g of biomass under magnetic stirring overnight in 10 mL of the solution, freezing at –18 °C and subsequently freeze-drying. Following these initial treatments, the sample was kept at room temperature.

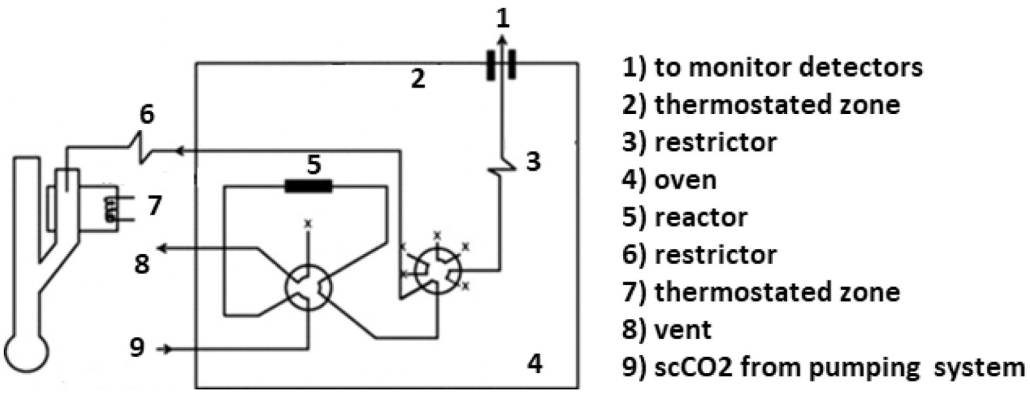

Fig. 2. Diagram of the laboratory system used for the $scCO_2$ treatment of microbial biomass.

Table 1
Yield in terms of purity of the biopolymer extracted from the biomass with pre-treatments.

Pretreatment	PHA purity (%,wt/wt)
Fine grind	68.8
Fine grind + H_2O	69.9
Fine grind + EtOH/H_2O	72.2
Fine grind + H_2O_2	86.6

Table 2
Influence of different $scCO_2$ operating parameters on PHA recovery: presence and type of co-solvent; extraction time; pressure and temperature of $scCO_2$.

T (°C)	P (MPa)	t (h)	co-solvent	PHA purity (%, wt/wt)	Recovery (%, wt/wt)
35	15	2	-	57.42 ± 4.49	82.35 ± 6.69
35	15	2	Methanol	59.38 ± 0.60	84.83 ± 3.88
35	15	2	PBS 5 % pH 7.4	60.77 ± 1.41	81.94 ± 5.40
35	15	4	PBS 5 % pH 7.4	61.78 ± 0.82	66.27 ± 0.29
35	15	2	PBS 5 % pH 7.4	60.77 ± 1.41	81.94 ± 5.40
35	15	1	PBS 5 % pH 7.4	57.58 ± 1.61	74.17 ± 1.44
35	15	2	PBS 5 % pH 7.4	60.77 ± 1.41	81.94 ± 5.40
35	20	2	PBS 5 % pH 7.4	62.53 ± 1.21	79.95 ± 3.61
40	15	2	PBS 5 % pH 7.4	71.58 ± 1.61	74.17 ± 1.44
40	20	2	PBS 5 % pH 7.4	75.13 ± 1.40	85.52 ± 6.38

2.4. Treatments of microbial biomass with $scCO_2$

A lab-scale stainless-steel Supercritical Fluid Extraction (SFE) reactor was used to carry out the cell lysis (Carlo Erba Instruments, Milan, Italy), with a cell reactor volume of 1 mL. Fig. 2 shows the diagram of the laboratory system used for the $scCO_2$ treatment of microbial biomass.

Liquid CO_2, stored under compression in a 30 kg cylinder, is pumped from a reservoir through a syringe pump (Carlo Erba Instruments) whose operation is controlled by dedicated software. The liquid CO_2 is compressed inside the syringe at a higher pressure at the critical pressure (72.9 bar). The syringe pump has an outer jacket refrigerated by a thermocryostat (Julabo F12, Seelbach, Germany) which avoids the gasification of CO_2. To this point, the movement of the piston inside the syringe is controlled via software when the target pressure value is reached. Reaching the target pressure inside the syringe pump, monitored by a pressure sensor, allows the CO_2 to subsequently reach the extraction cell housed in a thermostatic chamber heated to a temperature higher than the critical temperature ($T_C = 31$ °C). In this way, the CO_2 in the cell is under supercritical conditions.

The extraction can be carried out in static or dynamic mode: in the first case, the cell is filled to the available volume with the supercritical fluid for the necessary time for the extraction; in the second case, the supercritical fluid passes into the cell extracting it from the solid phase continuously. During the extraction process, the high-pressure extraction reactor allows the stabilization of the pressure inside. This can be monitored via a display on the syringe pump. Therefore, the reaction time starts automatically when the pressure has stabilized. This time is a few minutes. The cell lysis treatment involved the use of approximately 500 mg of dry biomass granules which were placed in the extraction cell. Two different co-solvents (MeOH and PBS phosphate buffer at pH 7.4) were tested, adding them in a 5 % v/v ratio to the sample. After the treatment, the sample was recovered from the cell and subjected to one or more washing cycles with H_2O and centrifugation (13.400 rpm at 5 °C for 20 min). The $scCO_2$ pressure was increased from 15 to 20 MPa (over critical value). However, we didn't increase the pressure up 30 MPa due to the possibility to stimulate the chemical degradation of the polymer. So, the optimal pressure value was 20 MPa. As far as the temperature chosen we experienced a range from 35 to 40°C with an extraction time range from 1 h to 4 h.

Table 3
Biomass treatments with hydrolytic enzymes, conducted at 37 °C and 50 °C.

T (°C)	enzyme	PHA purity (%, wt/wt)	Recovery (%, wt/wt)
50	CRL	57.42	82.35
37	CRL	59.38	84.83
50	PFL	60.77	81.94
37	PFL	61.78	85.27
50	trypsin	57.77	74.94
37	trypsin	60.58	81.17

Table 4
Biomass treatments with bromelain in single, sequential and combined action with CRL (at 50 °C).

Sequential treatment	PHA purity (%, wt/wt)	Recovery (%, wt/wt)
Bromelain	51.7	86.6
Bromelain + Bromelain	69.3	78.3
Bromelain + CRL	64.7	84.4

Table 5
Treatment of biomass with aqueous H_2O_2 solutions.

Time (h)	Concentration (%)	PHA purity (% , wt/wt)	Recovery (%, wt/wt)
4	30	81.61	41.67
4	20	81.67	45.51
18	10	71.89	34.65
18	5	81.91	68.23
18	2.5	52.22	35.82
18	1.25	96.27	87.69

2.5. Biomass treatments with hydrolytic enzymes

The protocol involved the use of 50 mg of a finely chopped freeze-dried biomass sample that was subjected to a series of treatments:

1) A preliminary treatment of the biomass was carried out in 1 mL of aqueous solution of the chosen hydrolytic enzyme (trypsin, lipase from *Candida rugosa*-CRL, lipase from *Pseudomonas fluorescens*-PFL or bromelain) at a concentration of 50 mg/mL for trypsin, CRL and PFL; 5 mg/mL for bromelain; and pre-set temperature (37 °C or 50 °C), in which the sample was kept under magnetic stirring for 24 h;

2) Subsequently, the sample was washed with 1.5 mL of distilled water for 30 min, centrifuged at 13.400 rpm at 5 °C for 20 min and stored at 4 °C.

2.6. Treatment with H_2O_2

100 mg of biomass were dried and subjected to three consecutive washings as follows:

1) The first washing was performed in 1.5 mL of H_2O_2 solution (in a concentration range between 1.25–30 %) in glass tubes equipped with screw caps and carried out under magnetic stirring at room temperature for a variable reaction time: overnight in the case of low reagent concentrations (1.25, 2.5, 5, 10 %), and 4–8 h in the case of concentrated solutions (20 and 30 %);

2) The second and third washings were conducted with 1 mL of H_2O for 30 min. The sample was centrifuged at 13.400 rpm at 5 °C for 20 min at the end of each washing step.

Table 6
Effect of the combined treatments on PHA purity and recovery.

Treatment	Purity (%PHA)	Recovery (%PHA)
$scCO_2$ + Bromelain	71.9	86.5
$scCO_2$ + trypsin	89.1	66.8
$scCO_2$ + H_2O_2 1,25 %	65.6	61.7
$scCO_2$ + H_2O_2 2.5 %	82.0	76.3
$scCO_2$ + H_2O_2 5 %	69.5	66.1
$scCO_2$ + H_2O_2 10 %	72.4	14.6
$scCO_2$ + H_2O_2 20 %	82.5	77.2
trypsin + H_2O_2 5 %	86.1	65.2
trypsin + H_2O_2 2.5 %	90.9	91.6
trypsin + H_2O_2 1.25 %	72.1	69.0
$scCO_2$ + trypsin + H_2O_2 1.25 %	92.2	92.3
$scCO_2$ + trypsin + H_2O_2 1.25 %	98.1	97.3
$scCO_2$ + trypsin + H_2O_2 1.25 %	88.9	70.9

Table 7
Effect of each treatment on the M_v of the PHA obtained.

Treatment	Viscometric molecular weight (M_v, kDa)
untreated PHA	116 ± 6
treatment with hydrolytic enzymes	127 ± 5
$scCO_2$	85 ± 2
treatment with H_2O_2	63 ± 1

2.7. Characterization of protein content by Bradford assay

The protein concentration of the different samples obtained from the treatment of the initial biomass with $scCO_2$, hydrolytic enzymes and H_2O_2 was determined according to Bradford assay, analyzing the supernatant obtained after the first washing with an aqueous phase of the treated biomass. A calibration line was created by measuring the absorbance of 1 mL dye solution at 595 nm, containing known dilutions in a range of bovine serum albumin (BSA) protein standards in the presence of Bradford reagent (1:1 v/v).

2.8. Purification and preparation of PHA film

An aliquot of 100 mg was taken from the previously treated dry sample, placed in a glassware equipped with a cap together with 50 mL of chloroform and 75 mL of distilled water. After 5 min of vortexing the mixture was transferred to a funnel for liquid-liquid extraction until reaching the separation of the two phases. The lower phase in chloroform, was collected and subsequently filtered with a glass syringe through PTFE filters, with a porosity of 0.45 μm, on a Petri dish and left to evaporate in a chemical fume hood overnight to obtain a PHA film.

2.9. Determination of the molecular weight

The viscosimetric molecular weight of recovered samples was evaluated on PHA solution in chloroform (0.6 g/dL) using a SCHOTT AVS 350 viscometer equipped with an AVS/SHT sensor; a LAUDA CD15 thermostatic bath set at 30 °C and a Ubbelohde capillary viscometer SCHOTT GERÄTE (diameter 0.46 mm). The M_v values were calculated according to a procedure published elsewhere [67].

2.10. Morphological characterization using scanning electron microscopy (SEM)

Prior to imaging, the PHA-rich biomass samples were freeze-dried to remove residual moisture. The dry samples were then mounted onto aluminum stubs using conductive carbon tape and sputter-coated with a thin layer of gold-palladium (~5 nm) to enhance surface conductivity and prevent charging under the electron beam. Scanning electron microscopy was used to obtain morphological information of the samples at the nanometric level, using an electron microscope with field emission source (FE-SEM) from ZEISS (model AURIGA 405), operating at low extraction voltage (1.5–4 kV) and current.

2.11. Determination of PHA purity and recovery

Poly(hydroxybutyrate-hydroxyvalerate) P(HB-HV) concentration was quantified by gas chromatography (GC-FID Perkin Elmer 8410) equipped with a glass packaged, 8 ft·1/8 in. (2 % Reoplex 400 coated Chromosorb GAW 60–80), N_2 was used as carrier gas, the oven temperature was initially maintained at 100 °C for 1 min; then increased up to 150 °C at a rate of 10 °C/min, the FID was maintained at 200 °C. Samples for PHA analysis were prepared as described elsewhere [68]. The relative abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was determined using as reference standard a commercial P(HB-HV) copolymer with 5 % (wt) HV content (Sigma - Aldrich, Milan). Italy). The 3HV content in PHA was reported as the weight ratio (wt%) of 3HV and (3HB + 3HV) monomers as (wt%). The purity and recovery were determined as described elsewhere [69].

All experimental data reported in Tables 1–7 present the standard deviation value calculated by using the following algorithm:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \mu)^2}{N}}$$

We considered the standard deviation as an expression of the variability of the measurement performed on the sample. On this basis, it was possible to obtain a significance of the experimental data.

3. Results

3.1. Pre-treatments on biomass containing PHA

As illustrated in Table 1, grinded biomasses samples were treated with the three different solutions selected for the pre-treatment and, among them, H_2O_2 was the most effective in enhancing biomass purity. The pre-treatment procedures weaken cell walls making the subsequent breakage steps easier (and more efficient) in releasing PHA granules from bacterial cells [70]. The oxidizing reagent H_2O_2 not only disintegrates the bacterial cell wall but also degrades all other cell constituents by oxidation [71], resulting in a higher efficiency.

In general, pre-treating biomass such as agricultural residues and food processing waste significantly reduces production costs, making PHAs a more viable alternative to conventional plastics [72,73]. Using ethanol and H_2O_2 can enhance the breakdown of complex polymers, making them more accessible for bioplastics production [74]. Pretreatment technologies can be categorized into physical, chemical, physicochemical and biological methods [75,76]. In this study, we first applied grinding as a physical pretreatment to reduce biomass size and crystallinity, which enhances hydrolysis by improving mass transfer and lowering crystallinity. Generally, the energy required for physical pretreatments depends on the desired particle size and degree of crystallinity reduction in the biomass. However, in cases where physical pretreatment is the only viable option, the energy demand often exceeds the theoretical energy content of the biomass, making the process costly and impractical for large-scale applications [77]. That is the reason we used a combination of pre-treatment methods to enhance the efficiency of the process. The combined pre-treatment of PHA-containing biomass (using ethanol, H_2O_2 and H_2O) can enhance the breakdown of complex polymers, making them more accessible for bioplastics production [78]. Ethanol can act as a solvent to dissolve certain biomass components, improving the extraction of valuable compounds. It is often used in combination with other methods to enhance efficiency [79]. H_2O_2 is a strong oxidizing agent that helps in breaking down lignocellulosic structures. Water helps in softening biomass and facilitating the enzymatic action [80,81].

3.2. Study of $scCO_2$ treatment of biomass containing PHA

The experiment was initially conducted at a pressure of 15 MPa, a temperature of 35 °C, and for a reaction time of 2 h. Then this experimental condition, in the static mode, was optimized both in the absence or with the addition of MeOH or PBS at pH 7.4 (5 % v/v). Table 2 shows the results obtained in terms of purity and recovery of PHA, in which the addition of co-solvent generally increases both PHA purity and recovery. On this basis, subsequent treatments of the biomass with $scCO_2$ were conducted always in static conditions, with the addition of PBS 5 % v/v by varying the biomass incubation time in the reaction cell (1 h, 2 h or 4 h). Change of time had no significant effect on PHA recovery and purity; it was agreed to choose the optimal incubation time as 2 h. A series of experiments were set up by changing the pressure and temperature of the extraction chamber. The results summarized in Table 2 confirmed that:

1) Pressure is one of the most important parameters in SFE, because it can drastically change the supercritical fluid properties. Generally, the increase of pressure positively affects the solvating power of the fluid. In our study, the increase of $scCO_2$ pressure (from 15 MPa to 20 MPa) at constant temperature of 40 °C increased the degree of purity and recovery percentage of PHA. However, higher pressures, e.g., 30 MPa, stimulate the chemical degradation of the polymer. So, the optimal pressure value was 20 MPa. 2) At optimal temperature and time, the increase in temperature has an enhancing effect on the kinetics of microbial inactivation since the treatment temperature is closely related to the mass transfer of the CO_2; increasing temperature enhances the diffusivity of the cell membrane, facilitating the diffusion of CO_2, as well as the extraction. In our study, the increase of temperature from 35 °C to 40 °C at the optimized pressure of 20 MPa resulted in an improvement of both purity and recovery obtained. For this reason, 40 °C was chosen as the optimal temperature.

In this study, we optimized pressure, temperature, time and used PBS, and methanol for biomass recovery in $scCO_2$. Each of these factors influences extraction efficiency, purity and yield, as briefly explained below [82]:

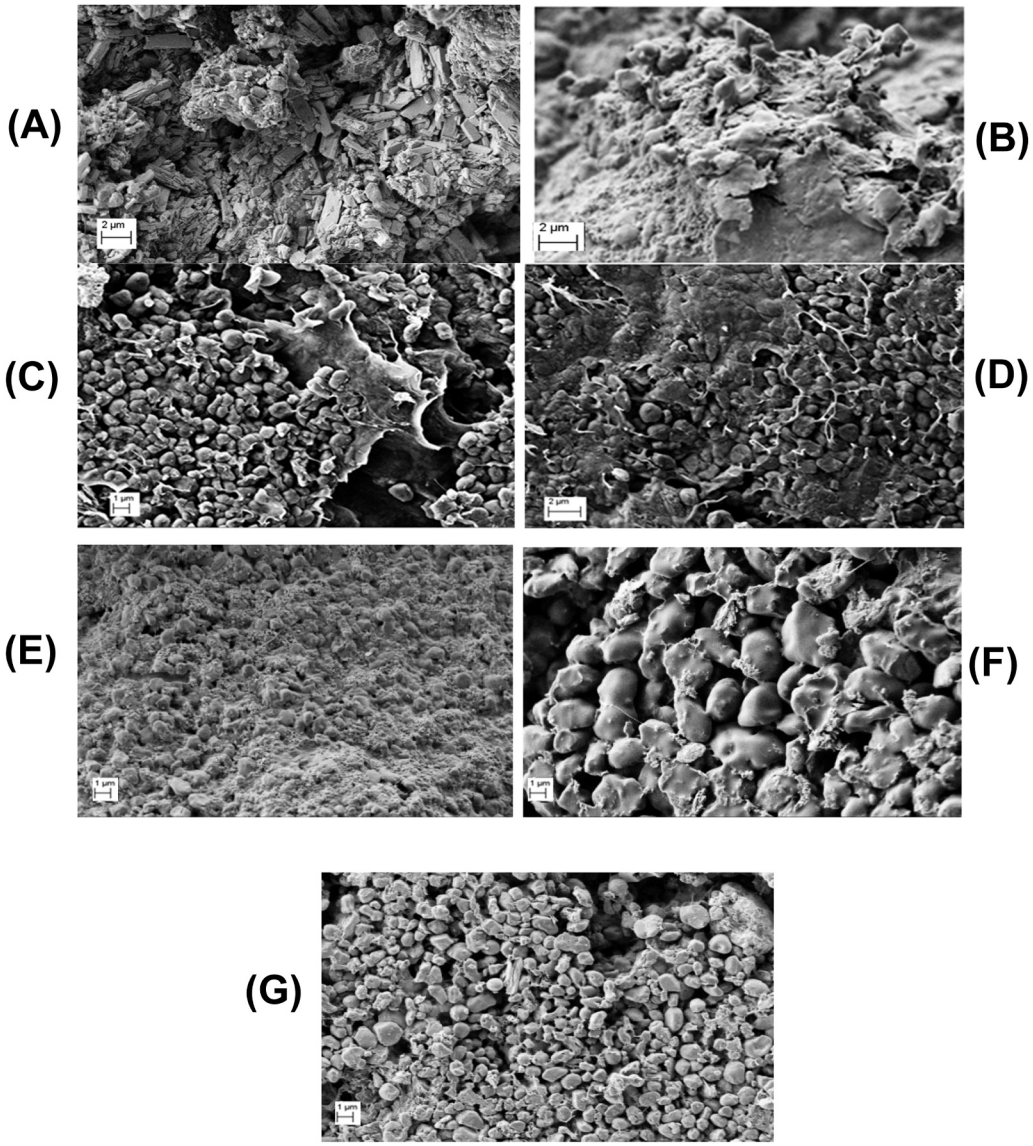

Fig. 3. (A) SEM of the untreated biomass (magnification = 10.00 K X); (B) SEM of the $scCO_2$-treated biomass (magnification = 15.00 K X); (C) SEM of the trypsin-treated biomass (magnification = 10.00 K X); (D) SEM of the trypsin-treated biomass (magnification = 15.00 K X); (E) SEM of the CRL-treated biomass (magnification = 10.00 K X); (F) SEM of the samples treated with H_2O_2 which contain the PHA granular structures (magnification = 25.00 K X); (G) SEM of the samples treated with H_2O_2 which contain the PHA granular structures (magnification = 10.00 K X).

1) Pressure: Higher pressures increase CO_2 density, improving solubility and extraction efficiency. Excessive pressure may lead to the unwanted co-extraction of impurities. Optimal pressure varies based on biomass type and target compounds [83,84].
2) Temperature: Elevated temperatures enhance mass transfer and reduce viscosity, improving recovery rates. Lower temperatures improve selectivity but may reduce overall yield [85,86].
3) Time: Longer extraction times allow for better diffusion and solubilization of biomass components. Excessive residence time may lead to thermal degradation or unwanted side reactions. Optimized time balances yield and purity [87].
4) PBS addition: PBS acts as a buffering agent, stabilizing pH during extraction. It may enhance biomass solubilization, improving recovery efficiency. Interaction with $scCO_2$ can affect polymer integrity and extraction kinetics [88].
5) Methanol addition: Methanol serves as a co-solvent, improving solubility and extraction efficiency. It enhances the recovery of biopolymers and bioactive compounds. Methanol concentration must be optimized to prevent unwanted degradation [89]. In the optimization step we tried to add an entrainer but no significant increase in purity and PHA extraction yield was obtained.

Studies suggest that moderate pressure and temperature, combined with optimized PBS and methanol concentrations, provide the best balance between efficiency and purity in biomass recovery.

3.3. Study of enzymatic treatments of PHA-containing biomass

Table 3 shows the effect of biomass treatment with different hydrolytic enzymes, evaluated at two incubation temperatures (37 °C and 50 °C). In all cases, PHA purity and recovery are higher at 37 °C, which might be due to the partial inactivation of the enzymes at 50 °C.

Table 4 shows the results relating to the use of bromelain at 50 °C; single, sequential, or combined treatment with CRL. In the case of sequential treatment with CRL, bromelain was used in the first step at 50 °C and, in the second step, CRL was used at 37 °C. The use of bromelain in combination with CRL allowed to obtain the best results in terms of average recovery and therefore it was used for subsequent sequential treatments of the biomass.

3.4. Study of H_2O_2 treatments of PHA-containing biomass

H_2O_2 treatment plays a significant role in PHA recovery from biomass, particularly in mixed microbial cultures [90]. H_2O_2 helps break down microbial cell walls, releasing PHAs while removing unwanted biomass residues [91]. Studies however show that excessive exposure to H_2O_2 can lead to polymer degradation, reducing molecular weight [92].

As reported in Table 5, the action of H_2O_2 generally allows to obtain a biopolymer with high purity rates (except the concentration of 2.5 %). However, the recovery results show operational problems for the concentration range of 2.5–30 %). The least limiting conditions can be achieved with 1.25 % concentration and by adopting longer contact times (18 h).

3.5. Study of multistep treatments of PHA-containing biomass

The different biomass treatment methods using green hydrolytic enzymes, H_2O_2 and $scCO_2$ were tested in multistep approaches, developing coupled treatments or subjecting biomass samples to all treatments in sequence. The extraction with $scCO_2$, in optimal operating conditions (40° C, 20 MPa, 2 h, PBS 5 %), is easily coupled to treatments with hydrolytic enzymes and with different H_2O_2 concentrations, where each process is followed by washing with H_2O. Table 6 show the best results obtained in such multistep treatments.

The implementation of a multiple recovery treatment of PHA from cellular biomass by inserting different physical, chemical and biological steps allowed us to obtain satisfactory results in terms of purity and recovery percentage. The coupled treatments allowed us to obtain higher recovery percentages and purity levels compared to the average of the results obtained from each single treatment.

3.6. Molecular weight evaluation of PHA extracted from biomass

The effect of the different biomass treatments (enzymes, H_2O_2 and $scCO_2$) on PHA molecular weight was evaluated. Enzymes such as proteases and lipases help break down non-PHA cellular components, improving purity [93]. Controlled enzymatic hydrolysis can preserve molecular weight [94], but excessive enzymatic activity may lead to polymer degradation [95,96]. H_2O_2 is used for cell lysis and oxidative purification, helping remove unwanted biomass residues [97]. High concentrations or prolonged exposure can reduce molecular weight due to oxidative degradation [98]. $scCO_2$ enhances PHA solubility and purification without requiring toxic solvents. Pressure and temperature conditions must be optimized to prevent polymer degradation while maximizing recovery.

The results of the viscometric average molecular weights, reported in Table 7, highlight that each treatment has a different impact on the structure of the extracted biopolymer.

Hydrolytic enzymes act as mild digesting agents for PHA recovery, and they do not decrease the M_v of the polymer [99]. However, for the $scCO_2$ method, sometimes a degradation of the polymer may occur, due to the extreme conditions used [100]. Regarding H_2O_2, it caused the highest degradation, resulting in the lowest molecular weight among all treatments. So, for the single use of each method, there should be a good balance among M_v, recovery and purity [101]. Studies suggest that combining enzymatic treatments with $scCO_2$ extraction can improve PHA purity while maintaining molecular weight integrity.

3.7. Morphological characterization of the biomass

Fig. 3 (A-G) show the images obtained by SEM analysis of different samples of microbial biomass before and after treatment for PHA recovery. Fig. 3A presents the high complexity of untreated biomass, rich in crystalline structures that can indicate an abundance of salts. The microbial cells are indistinguishable from the abiotic fraction.

Conversely, the $scCO_2$-treated sample is more homogeneous, and the biomass is visibly composed of cellular debris and free filamentous components (Fig. 3B). Coupling the $scCO_2$ treatment with hydrolytic enzymes (specifically with trypsin), it is possible to see many granules with variable size: these could be the structures of intracellular accumulation of the biopolymer (Fig. 3C,D). The same type of granular elementsis also distinguishable in the biomass treated with CRL, but in this case they appear immersed in an amorphous matrix (Fig. 3E). In the SEM images of samples treated with H_2O_2, the PHA granular structures are clearly seen (Fig. 3F,G).

4. Conclusions

This study demonstrated the extraction of PHA from microbial biomass with a developed protocol that is based on the disruption of cells by combining $scCO_2$ + enzymes + H_2O_2. The effects of time, pressure, temperature and co-solvent (MeOH or PBS) were investigated. Based on our results, this new approach could increase the purity and recovery of PHA above 95 %. The specific combination of these three methods allowed us to preserve the molecular weight of the PHA, shown by the viscometric molecular weight results. So this green multistep approach (combination of three green techniques) can be considered as a valid alternative to chlorinated solvents, identifying green chemistry as a turning point for the industrial production of biopolymers starting from a complex biomass. Based on the promising results of this green multistep extraction protocol, future studies could pursue several avenues to further optimize and expand its applicability:

1) Scale-up and industrial translation: Assessing the feasibility and efficiency of the combined scCO$_2$–enzyme–H$_2$O$_2$ process in pilot and full-scale production environments, including cost analysis and throughput assessment.
2) Strain and biomass diversity: Evaluating protocol performance across different microbial strains and feedstocks to determine universality and tailor process parameters accordingly.
3) Process sustainability metrics: Investigating life cycle impacts, energy consumption, and reagent recovery strategies to quantify the ecological footprint compared to conventional methods.
4) Polymer quality control: Exploring long-term stability, crystallinity, and mechanical properties of the extracted PHA under various post-treatment conditions.
5) Enzyme engineering and synergy: Engineering more resilient or specific enzymes and studying synergistic effects with oxidizing agents to enhance cell disruption.
6) Integration with downstream processing: Linking this green extraction with purification, formulation, and application-specific steps for medical, packaging, or agricultural-grade PHAs.

CRediT authorship contribution statement

Gaia Salvatori: Formal analysis, Data curation. **Farid Hajareh Haghighi:** Writing – original draft. **Laura Lorini:** Investigation, Conceptualization. **Sara Alfano:** Formal analysis, Data curation. **Cleofe Palocci:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Francesco Valentino:** Investigation, Conceptualization. **Laura Chronopoulou:** Writing – review & editing, Supervision, Methodology. **Marianna Villano:** Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cleofe Palocci reports was provided by University of Rome La Sapienza. Palocci Cleofe reports a relationship with University of Rome La Sapienza that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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