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A novel sustainable PHA downstream method

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The pernicious impact of plastic originating from petroleum-based sources has been an increasing threat to our environment. Amongst several strategies tested and implied to manage these plastic wastes, one promising alternative is switching to biodegradable polymers. Polyhydroxyalkanoates (PHAs) are a class of microbial polyesters that are biodegradable, biocompatible and an attractive alternative for future packaging applications. They offer an approach to carbon neutrality and support a more sustainable industrialization. However, as these microbial polymers are stored intracellularly, development of downstream processes for extraction and purification are essential and are the most cost-intensive steps. A major drawback in most of the existing state-of-the-art is the use of harsh organic solvents which contributes to the non-eco-friendliness of the processes. This work proposes an alternative, sustainable PHA downstream process comprising the use of a green solvent. Biomass with an intracellular PHA content of ca. 49% (gPHA/gVSS) produced by mixed cultures was used in this study. A new hydrophobic natural deep eutectic solvent (hydrophobic NADES), Thymol:Vanillin (8:2 molar ratio) was synthesised and used as the extraction solvent while 1-heptanol was used as the purification solvent. Also, different intensities of sonication were applied to assist the cell wall rupture for the efficient release of PHAs. The optimised process yielded ~99% polymer purity and recovery of ~42% and did not alter the polymer composition. Furthermore, after successful extraction and purification of PHA, the organic solvents (hydrophobic NADES and 1-heptanol) used can be recycled or reused as per end-user requirements.

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Introduction

Plastics are a ubiquitous part of our modern-day life due to their manifold characteristics like low cost, lightness, flexibility, non-degradability and stability. As per the records of the Association of Plastics Manufacturers in Europe, global plastics manufacturing attained 370 million tons in 2020.¹ Moreover, the larger share of plastics used in diversified sectors like households, pharmaceuticals, industrial production, etc., are derived from petroleum-based crudes.² These long-chain, complex structured hydrocarbons are resistant to degradation and produce toxic by-products upon incineration, thereby imparting an adverse effect on the environment (both land and sea).³ Also, these conventional plastics pose an immi-

nent environmental threat by accumulating in sea and land.⁴ These concerns promote the need for greener alternatives that need to be bio-degradable, bio-compatible and exhibit competitive physicochemical and mechanical properties as its counterparts.⁵

The quest for eco-friendly biodegradable alternatives guided the research to several types of bioplastics, more particularly to polyhydroxyalkanoates (PHAs). PHAs are a family of microbial thermoplastic polyesters composed of several *R*-hydroxyalkanoates and recognised as prokaryotic storage granular inclusions in the cell's cytoplasm for energy and carbon source.⁵ PHA's copolyesters, namely polyhydroxy(butyrate-co-valerate) (PHBV) (Fig. 1), are considered viable and outstanding bio-based materials to replace the conventional petro-

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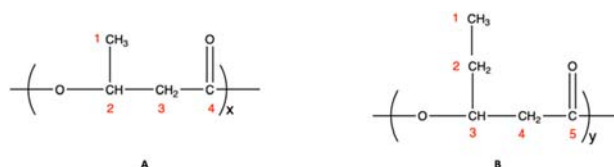


Fig. 1 Chemical structure of monomers of PHBV (A) hydroxy butyrate (HB) (B) hydroxy valerate (HV). (The numbers on each carbon atoms are used to identify them as peaks in respective NMR graphs, in Fig. 7 and 8).

chemical plastics (*e.g.*, polyethylene, polypropylene, and ethylene terephthalate) not only due to their mechanical and thermal properties, but also their astonishing chemical characteristics, namely biocompatibility and biodegradability.^{2,6,7}

According to the directive (EU) 2019/904 of the European Parliament and the Council of 5 June 2019, global bioplastics manufacturing is increasing.⁸ Furthermore, we have witnessed an exponential growth in the number of relevant studies with promising findings regarding PHA production and its pertinent applications.^{9–12}

Although PHA manufacturing is multi-faceted, and it possesses advantages that outperform synthetic plastics, its industrial production remains limited due to its considerably high production cost, as opposed to conventional fossil-fuel-based plastics (1.18–6.12 € per kg *vs.* <1 € per kg).^{13,14} The paramount reasons behind the mentioned economic disadvantages are the high cost of the fermentation process (carbon feedstock) and the downstream processing, where the latter accounts for almost 50% of the polymer's production costs.^{10–12,15} The downstream of PHA is particularly challenging because PHA is accumulated as intracellular granules, requiring an extra processing step of disruption/destabilization of PHA-rich biomass membranes.¹⁶

The downstream processing of PHA broadly comprises the following steps: PHA-rich biomass separation from the supernatant (by means of flocculation, decantation, centrifugation, lyophilization and/or filtration), biomass pre-treatment to weaken and disrupt the cell wall and enhance PHA extraction step (*e.g.* mechanical pre-treatment, heat, alkali and oxidants, enzymatic methods), PHA extraction and purification.^{14,17,18}

The PHA extraction has been widely studied and can be grouped into 2 major categories: (1) digestion of non-PHA cell mass (NPCM) using chemicals,¹⁹ enzymatic,²⁰ biological,²¹ or mechanical disruption²² methods or (2) solvent-based extraction methods, which involves cell permeability modification and subsequent release and PHA solubilization.¹⁸ To date, the most efficient method for extracting PHA from the cell biomass is solvent extraction. This approach presents undoubted advantages regarding its simplicity, high recovery yields and purity, and negligible quality degradation. Furthermore, this method enables bacterial endotoxins LipoPolySaccharides (LPS) removal, improving the polymeric quality for medical applications.^{18,23}

Chlorinated solvents, such as chloroform,^{24–26} 1,2-dichloroethane,²⁵ methylene chloride^{25,27} and sodium hypochlorite²⁸ have been successfully applied for PHA extraction, with chloroform being the benchmark protocol for PHA extraction. Nevertheless, chlorinated solvents have a harmful character both for the environment and humans. Also, such processes involve significant capital investment and operation costs not only because of the high amount of toxic and volatile solvents required but also the energy employed. After chloroform extraction, the PHA purification involves the addition of large amounts of a PHA “non-solvent”, commonly around 10 volumes of ethanol per volume of PHA solution, to precipitate the PHA.^{18,29}

Many sustainable alternatives to chlorinated compounds for solvent extraction have been proposed in the literature (*e.g.*, ethyl acetate;^{30,31} propylene carbonate;^{31,32} 1,3-dioxolane;³³ Anisole;³⁴ dimethylcarbonate (DMC);^{35–37} supercritical fluids (SC-CO₂);³⁸ ionic liquids^{39,40}). Even though some of these proposed methodologies presented promising results concerning extraction and purification yields, they lack economic competitiveness. For instance, cyclic and linear carbonates like propylene and ethylene carbonates require high temperatures for efficient PHA extraction, which on the one hand, is energy-demanding and, on the other, may be detrimental to the quality of the final polymer due to a reduction of polymer molecular mass. Although ionic liquids and supercritical fluids extraction are considered cutting-edge technologies, yet their implementation is energy intensive and not cost-effective. Also, most of the organic solvents used for extraction or purification of PHAs cannot be reused or recovered, shunning the process from being sustainable.

Other extraction processes, such as enzymatic, biological, chemical and also mechanical approaches have been studied to mitigate the impact of these harmful solvents on the environment.^{41–47} Unfortunately, these approaches also exhibit some disadvantages. Regarding the enzymatic and biological methods, although these methods allow the maintenance of polymer molecular mass and display satisfactory recovery yields, their procedure is intricate, involving several sequential steps and therefore extending the extraction process. Moreover, in the case of enzymatic disintegration, the cost of the pure/cocktail of enzymes still drives up the overall extraction cost. Also, a wide range of surfactants (*e.g.*, sodium dodecyl sulphate (SDS),¹⁹ switchable anionic surfactants (SAS),⁴⁸ *etc.*) display technical simplicity and can reach high extractions when complemented with several pre-treatments (*e.g.*, sodium hypochlorite); however, the required wastewater treatment (for PHA recovery, purification, and recycle) and the relatively high cost of chemical digesters turns the process expensive and not environmentally friendly.²¹ The use of alkaline compounds, such as sodium hydroxide (NaOH), was successfully applied for PHA extraction as a green alternative to chlorinated compounds.⁴⁹ However, in some studies, NaOH digestion caused degradation of the biopolymer, namely reduction of its molecular weight.^{50–53} Mechanical approaches (*e.g.*, bead milling) do not involve any chemicals, but they are not an economically attractive process due to the high capital investment cost, long processing time, precise control of various process parameters, and difficulty in scaling up.⁴⁶

Deep eutectic solvents (DESs) are a recent class of green solvents that exhibits 100% atom economy, negligible environmental toxicity, tunability, biodegradability and are cheap to manufacture.^{54,55} They are synthesised mainly by mixing two or three components that are self-associated through hydrogen bond interactions. The word deep implies a considerable drop in the melting point of the eutectic mixture with respect to its individual components. Being eco-friendly and cheap solvents, DESs gained popularity for myriad applications including extraction/purification.⁵⁶

DESs can be broadly classified into two types, hydrophilic and hydrophobic, based on their affinity to water. Hydrophilic DESs are extremely unstable in aqueous media as water's hydrogen bonding capacity greatly inhibits the existing hydrogen bonding in DESs.⁵⁷ The challenge of extracting substances that are insoluble in complex polar media, such as PHA, may be addressed by the recently exploited sustainable hydrophobic DESs.⁵⁸ Exploration of a new hydrophobic natural deep eutectic solvent (hydrophobic NADES) system for the extraction of PHA sets the premise for this work.

The aim of the work was to design a sustainable PHA downstream process with a high PHA recovery efficiency and a high PHA purity, using green solvents, which can be recycled/reused. The novelty of this work relies on the extraction of PHA using a hydrophobic NADES, comprising thymol and vanillin. To the best of our knowledge, this NADES is a novel solvent and has been applied for the first time for the recovery of PHA from microbial biomass. After the synthesis and characterization of the proposed hydrophobic NADES, a downstream process from a PHA-enriched microbial culture was developed, including the PHA extraction and purification steps with non-hazardous solvents. Furthermore, the organic solvents used for extraction and purification were either recycled or reused for its intended usage in fragrance and flavour industries.

Experimental

Materials

Analytical grade thymol (>98.5% purity) and vanillin (>97% purity), used for the preparation of NADES, were obtained from Sigma Aldrich, Portugal. Pure, solid PHA from YPACK project (<https://www.YPACK.eu>), P(HB 77 C mol%, HV 23 C mol%), with a purity of 86.1% (w/w) was used for the PHA solubility tests. Reference extraction solvent was chloroform (99.8% purity, from Honeywell), used for PHA solubility tests and as the extraction solvent. The PHA-containing biomass was produced at the pilot-plant scale at UCIBIO (Caparica, Portugal) using mixed microbial cultures (MMCs) and cheese whey as feedstock within the scope of the YPACK project. Solvents used for the purification of PHA were ethanol (96% purity, from Carlo Erba Reagents, France), acetone (100% purity, from Carlo Erba Reagents, France), isoamyl alcohol (98% purity, from Sigma Aldrich, Portugal) and 1-heptanol (99% purity, from Merck, Germany). The filter papers, (coffee filters, grade 2 and grade 4) procured from Auchan, Portugal, were used in the downstream process.

Procedures

Synthesis and characterization of a new hydrophobic natural deep eutectic solvent (hydrophobic NADES). The pure NADES was synthesised by combining different known molar masses of thymol and vanillin in sealed screw glass vials to prevent mass loss and avoid any contamination. The samples were mixed at different molar ratios. Next, the mixture was stirred magnetically at 300 rpm for 1.5 hours at 81 °C using an oil

bath. Once the solid powder turns into a uniform liquid mixture with no apparent suspensions, NADES was allowed to cool down gradually and was observed overnight at 30 °C to determine its stability. The selected extraction solvent was the NADES system, comprising of a definite molar ratio of thymol and vanillin, exhibited minimised viscosity-density and had lower cost. Also, physical properties like density, viscosity, melting point, thermal degradation, water content of this NADES were characterised as described in subsection "Characterization techniques for the synthesised NADES"

PHA solubility tests in selected NADES and in chloroform

NADES. To understand the maximum solubility of pure PHA polymer in NADES and its temperature correlation, the amount of polymer was dissolved in NADES, starting from 5% (w/w) and gradually increased in multiples of 5 (e.g., 10%, 15% (w/w) and so on). Initially, the solution was stirred at 25 °C and 300 rpm for 12 hours. In case of complete dissolution, further PHA was added. And if there is incomplete dissolution, the temperature was increased until complete dissolution was observed. As the mass of the polymer was increased, the temperature was increased gradually up to 75 °C to solubilize the polymer while the stirring rate and time were kept constant.

Chloroform. Similar strategy was employed to evaluate the maximum solubility of pure PHA in chloroform, the benchmark for PHA extraction. Starting at 1% (w/w) and gradually increasing, PHA was dissolved in CHCl₃ in sealed glass vials. The solution was stirred at 300 rpm at 37 °C using an oil bath.

PHA-containing biomass pre-treatment. The schemes of the downstream process for PHA extraction and purification, both the benchmark procedure and the one proposed in this work, are shown in Fig. 2. The PHA-containing biomass pre-treatment comprises the following steps, based in Duque *et al.*:⁵⁹ (i) the biomass was acidified by adding H₂SO₄, to a pH value of 1–2, at the end of the PHA accumulation stage, to stop the bioactivity of the PHA-containing biomass; (ii) the biomass was concentrated, by decantation under cold conditions (~4 °C); (iii) cycles of water washing-centrifugation (10 000g for 15 min at 4 °C, Sigma 4-16KS, Germany) and (iv) lyophilised (ScanVac, CoolSafe), for the total removal of water content and stored in a desiccator for further experiments and under dark conditions.

PHA extraction with chloroform and purification with ethanol. The chloroform extraction was based on Duque *et al.*⁵⁹ Briefly, PHA-containing biomass pre-treated (lyophilised) was suspended in chloroform (in a ratio of ca. 50 mL CHCl₃ per 1 g lyophilised material or 2% (w/v) material per solvent) in sealed screw glass vials immersed in an oil bath, with continuous stirring for 72 h at 37 °C. Thereafter, the PHA suspension was filtered with a coffee filter (grade 4) to remove all undissolved material (as shown in Fig. 2).

In some cases, the resultant extracted PHA solution was purified, based in Palmeiro-Sánchez *et al.*,⁶⁰ by adding 10 volumes of ethanol under cold conditions (~20 °C) per volume of CHCl₃. The precipitate was separated from the liquid phase by filtration with a coffee filter (grade 4). The filtrate was dried

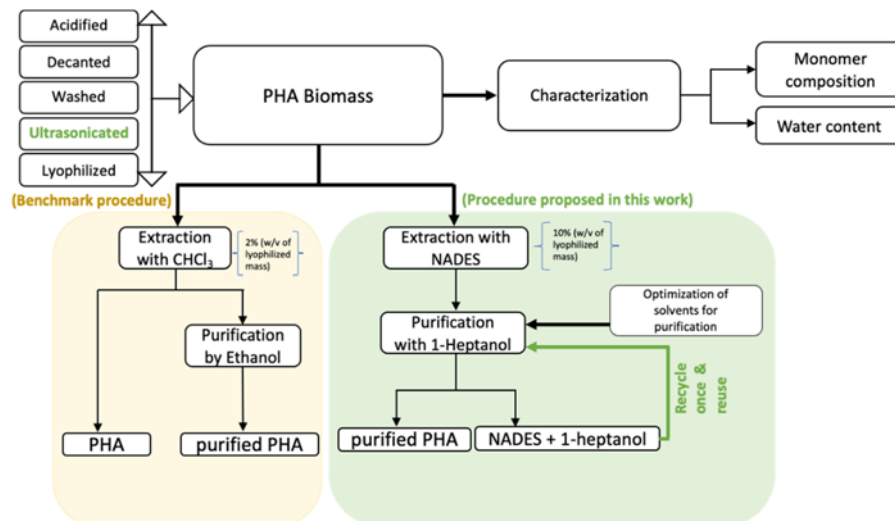


Fig. 2 Scheme representation of the downstream process for PHA extraction and purification, following the benchmark procedure and the sustainable procedure proposed in this work.

in the fume hood, allowing the pure PHA to be recovered as a powder.

When the PHA extraction was not consistent in terms of reproducibility of the purity, the pH-neutralised PHA-containing biomass would be suspended in water and processed by ultrasounds, with an ultrasonic processor (Vibra Cell Sonics, VCX750, USA). A high-grade titanium alloy Ti-6Al-4 V probe with $\frac{1}{2}$ inch diameter was used to sonicate the samples at 20 kHz frequency for 15 min, at 1 s pulse on and 1 s pulse off mode, with 40%, 50% and 60% amplitude. The PHA biomass was dipped in an ice bath while sonication to keep a controlled temperature of <26 °C.

PHA extraction with NADES and purification method with several solvents. PHA-containing pre-treated biomass (lyophilised) was suspended in NADES (in a ratio of 0.05, 0.1 g & 0.15 g of lyophilised material per g of NADES, corresponding to 5% (w/w), 10% and 15% (w/w) material per solvent) in sealed screw glass vials immersed in an oil bath, with continuous stirring for 48 h at 37 °C. Thereafter, the PHA suspension was filtered with a coffee filter (grade 4) to remove all undissolved materials.

To purify the PHA, several solvents were studied, namely ethanol, acetone, isoamyl alcohol and 1-heptanol. In the case of purification with ethanol and acetone, since they were more volatile under ambient conditions, they were kept in the refrigerator (-4 °C) before adding to the extract filtrate. For isoamyl alcohol and 1-heptanol, less volatile than previous solvents, the resultant PHA solution was purified by adding certain volumes of each solvent per volume of extracted filtrate and stirred at 250 rpm for 15 min at room temperature.

Afterwards, the PHA precipitate was recovered by filtration with a coffee filter (grade 2). The PHA retentate was dried in the oven (60 °C, 6 hours) to remove the remaining solvent residues.

Recovery of the organic solvents. In the downstream process proposed, while we used non-hazardous solvents, it was also

important to minimize the wastage of the organic solvents. After the extraction and purification, PHA was collected on a filter as retentate. A protocol was proposed to recycle or reuse the filtrate consisting of mixture of NADES and optimised purification solvent.

Characterization techniques for the synthesised nades

Thermal properties

Differential scanning calorimetry. The melting point of the NADES was characterised using differential scanning calorimeter (DSC 131, Setaram, Caluire, France). The sample was placed in an aluminium crucible and cooled from 25 °C until -35 °C and then heated up to 40 °C at 1 °C min^{-1} ramp and again cooled to -35 °C at 5 °C min^{-1} ramp.

Thermogravimetric analysis. Thermogravimetric analyses (TGA) were performed to check the thermal stability of the NADES. TGA Q-50 analyser (TA Instruments, USA) was used to determine the degradation temperature using a ramp of 5 °C min^{-1} , in the temperature range of 25–300 °C under a nitrogen atmosphere with a flow rate of 40 mL min^{-1} on balance side and 60 mL min^{-1} on sample side. The approximate sample weight of 10 mg was placed into an aluminium sample pan. Purity of the NADES was studied from % loss of mass under inert atmosphere in the range 25–300 °C from the TGA curves.

Density and viscosity

The densities and viscosities were measured with an automated SVM 3000 Anton Paar rotational Stabinger viscometer-densimeter (Graz, Austria). The measurements were performed in the temperature range of 10 °C to 80 °C at atmospheric pressure. Further details on working principles and uncertainties of the measurements can be found in work of Tariq *et al.*⁶¹

Water content

The hydrophobicity of the NADES was determined by measuring the saturated water content. One mL of NADES was added in a glass vial and 1 mL of water was dropped on it. The NADES was kept in contact with water at 30 °C for 12 hours. Then 400 μL of NADES was carefully pipetted from the bottom of the vial and its water content was measured using a Karl-Fischer coulometer with a diaphragm (Metrohm®, model 831 KF coulometer, Herisau, Switzerland).

Characterization techniques for the pha samples

Determination of PHA monomeric content in the biomass by GC-FID

Samples (lyophilized PHA-containing biomass, extracted PHA biomass using both CHCl_3 and NADES and purified PHA samples) were characterized in terms of PHA content, PHA purity and polymer composition by gas chromatography with a flame ionization detector (GC-FID) as described by Duque *et al.*⁵⁹ Briefly, 2–4 mg of lyophilized PHA-containing biomass, extracted PHA, or purified PHA was combined with 1 mL of acidified methanol (20% sulphuric acid) and 1 mL of chloroform with heptadecane (HD, concentration of approximately 1 g L^{-1}) as internal standard. Samples were digested in heating block throughout 3.5 h at 100 °C, in pressure resistant glass tubes. After digestion, the mixture was cooled to room temperature. Subsequently, 1 mL of distilled water was added to the mixture and vortexed for 1 min. The organic phase containing the methylated monomers formed at the bottom layer was extracted with glass pipettes. Approximately 1 mL of the organic phase was withdrawn into GC vials for analysis. 200 μL of the sample was injected into a gas chromatograph equipped with a Restek column (60 m, 0.53 mm internal diameter, μm film thickness) and a flame ionization detector, using carrier gas (Helium) at 1.0 mL min^{-1} . Samples were analysed under a temperature range starting at 40 °C, increasing to 100 °C at a rate of 20 °C min^{-1} , to 155 °C at a rate of 3 °C min^{-1} and finally reaching to 220 °C at a rate of 20 °C min^{-1} for ensuring cleaning of the column after each run. The temperature of the GC injector and detector was set at 300 °C and 230 °C, respectively. The monomers 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) concentrations were determined using two calibration curves, one for 3HB and other for 3HV, using standards of a commercial copolymer P(3HB-co-3HV) (88:12 mol% 3HB:3HV, Sigma-Aldrich, Burlington, MA, USA), ranging from 0.1 to 6 g L^{-1} .

Determination of molecular weight of purified PHA by gel permeation chromatography (GPC)

The polymer was dissolved in chloroform (0.2%–0.4% m/v) at room temperature. Then, the solution was filtered with a

regenerated cellulose filter 0.22 μm and analysed by Gel Permeation Chromatography (Smartline, Knauer, Germany) using a Phenogel Linear column 300 $\times$ 4.6 mm (Phenomenex, USA), using chloroform as mobile phase and a flow rate of 1 mL min^{-1} . The operating temperature was 30 °C. Samples were stored at 4 °C before injecting 100 μL in the GPC circuit. A RI detector (Smartline 2300, Knauer, Germany) was used for polymer detection. The relative molecular weight (MW) of the polymers was determined by adopting monodisperse polystyrene (PS) standards (SUPELCO) with MW ranging between 2520 kDa and 0.370 kDa. GPC Clarity software relying upon the universal calibration method has been adopted to calculate the relative MW of PHA. A 12-point calibration curve in correlation with poly(3-hydroxybutyrate) (P(3HB)) was formulated using the Mark–Houwink–Sakurada relationship, $[\eta] = K \times M^\alpha$, where $[\eta]$ is viscosity number limit and ‘K’ and ‘ α ’ are the Mark–Houwink constants for each solvent/polymer/temperature system.⁶² The values of these constants at 30 °C used for the pairs P(3HB)/chloroform and PS/chloroform, were respectively $K = 11.8 \times 10^3 \text{ mL g}^{-1}$, $\alpha = 0.78$ and $K = 7.16 \times 10^3 \text{ mL g}^{-1}$, $\alpha = 0.76$.⁶³ Sample injection volumes of 150 μL were used.

Nuclear magnetic resonance (NMR)

Extracted and purified PHA samples using CHCl_3 and NADES were characterised in terms of type of polymer (homopolymer or co-polymer), polymer integrity and 3HB:3HV monomeric composition by NMR technique. The NMR spectra were acquired in a Bruker AVANCE III 400 spectrometer which operated at a proton frequency of 400.15 MHz (^1H) and a carbon frequency of 100.61 MHz (^{13}C). A 5 mm QNP probe head was used, and the temperature was set to 298 K for all the experiments. The proton and carbon chemical shift were calibrated relative to chloroform (internal reference, δ 7.26 ppm and internal reference δ 77.00 ppm, respectively).

The experimental conditions for the acquisition of ^1H NMR – spectral window of 8012 Hz (20 ppm) centred at 400 Hz, 64 transients were acquired with a relaxation delay of 1 s.

The experimental conditions for the acquisition of ^{13}C NMR – spectral window of 24 038 Hz (238 ppm) centred at 10 060 Hz, 1024 transients were acquired with a relaxation delay of 2 s.

Numerical analysis

PHA content in the biomass (wt%, gPHA/gVSS) was determined in terms of percentage of volatile suspended solids (VSS), on mass basis, considering VSS to be constituted on active biomass and PHA, as follows:

$$\text{PHA content}(\%, \text{g g}^{-1}) = \frac{\text{mPHA}}{\text{mVSS}} \times 100 \quad (1)$$

where, mPHA is the mass of PHA (m of 3HB + m of 3HV quantified by GC) and mVSS (g) is the mass of VSS in the lyophilised sample used for the GC analysis.

PHA purity ($\%, \text{g g}^{-1}$) was calculated by dividing the mass of PHA extracted quantified by GC by the mass

of the final extraction product sample used for the GC analysis.

PHA recovery yield (% , g g^{-1}) was calculated as follows:

$$\text{PHA recovery (\% , g g}^{-1}\text{)} = \frac{\text{mPHA}_{\text{extracted}}}{\text{mPHA}_{\text{cell}}} \times 100 \quad (2)$$

where $\text{mPHA}_{\text{extracted}}$ (g) is the PHA mass of the final extraction product and $\text{mPHA}_{\text{cell}}$ (g) is the PHA mass content of the biomass from which the final product was extracted.

All results were representative of three independent experiments and are presented as the mean value $\pm$ standard deviation.

Results and discussion

Synthesis of the hydrophobic natural deep eutectic solvent (hydrophobic NADES)

Different hydrophobic NADES systems were prepared according to the method described in the “Experimental section”. Thymol and vanillin were mixed in 9 different molar ratios of 1 : 9 to 9 : 1 respectively. As it can be observed from the Fig. 3A, a higher quantity of thymol led to successful formation of NADES. Based on viscosity, density and cost, NADES with 8 : 2 (thymol : vanillin) molar ratio was chosen for the downstream processing. Lower viscosity of NADES was needed to reduce operational energy consumption; lower density was needed to ensure complete and facile dissolution of lyophilised PHA powder. The optimised NADES formed a homogeneous mixture with slightly yellow colour, as depicted in Fig. 3B.

Characterization of the hydrophobic natural deep eutectic solvent (hydrophobic NADES)

As seen in Fig. 3A, although 1 : 1 of thymol and vanillin led to apparent formation of NADES, this was found to be *meta*-stable. While measuring density-viscosity at temperatures $<25^\circ\text{C}$, they exhibited quick crystallisation and hence, this was not considered for further investigation. Viscosities and densities of the remaining successful NADESs were measured (see Fig. 4A and B). It was observed that the higher the concentration of thymol in NADES, the lower was the viscosity and density of the NADES. Considering that thymol is more expensive than vanillin, and NADES with molar ratios 8 : 2 and 9 : 1 exhibited comparable rheological properties, 8 : 2 molar ratio was chosen for pure PHA solubility studies for relatively lower cost.

Fig. 4C depicts the thermal degradation profile of the optimised NADES. It can be seen for the graph that at 100°C , there is very insignificant loss of mass ($<0.5\%$), which hints at the low water content in the NADES. Also, we can see from the graph that there is no degradation of the solvent (*i.e.*, NADES) in the temperature range where the downstream process is operated and beyond it. Thus, there remains a scope for further increase in the operational temperature for even enhanced recovery of PHA. However, that was beyond the scope the present study.

Fig. 4D exhibits the differential scanning calorimetry curve of the optimised NADES for determining the solid-liquid phase behaviour along with the variation of temperature. The individual components of the NADES, thymol and vanillin has a melting point of 51°C and 81°C respectively.⁶⁴ But it can be seen in Fig. 4D that, for the NADES, as the temperature is ramped up from -36°C through to 40°C , a clear decline in heat flow was observed between 33.86°C and 15.38°C . The maximum decline of heat flow happened at $\sim 26^\circ\text{C}$, indicating the melting point of the optimised NADES. Since the melting point of the solvent is much lower than its individual components, it can be rightly classified as a deep eutectic solvent.

Also, the water content of the optimised DES was found to be $<0.5\%$ (w/w), thus, it can be categorised as a hydrophobic solvent.⁶⁵

Pure PHA solubility tests in selected NADES & chloroform

At first, pure PHA with an 3HB : 3HV monomeric content of 77 : 23% Cmol basis was suspended in NADES (5% PHA (w/w)) and was allowed to dissolve overnight at 30°C . Depending on visual appearance of the consequent solution, two approaches were established: (i) if the consequent solution was clear, without suspended particles, the PHA concentration was increased; (ii) alternatively, if particulate suspension was noticed, the temperature was increased to dissolve the particles. The maximum measured weight % of PHA dissolved at a particular temperature is given in Table 1.

As observed in Table 1, the solubility of PHA in NADES increases as the temperature is increased (tested up to 75°C). However, a maximum solubility of 35% (w/w) of PHA in NADES at 60°C was observed. No further dissolution was possible, even after increasing the temperature. Since, increase of viscosity of the solution would negatively impact the fluidity of the downstream process, an optimum temperature value of 37°C was chosen (within the optimised range of 30°C – 40°C).

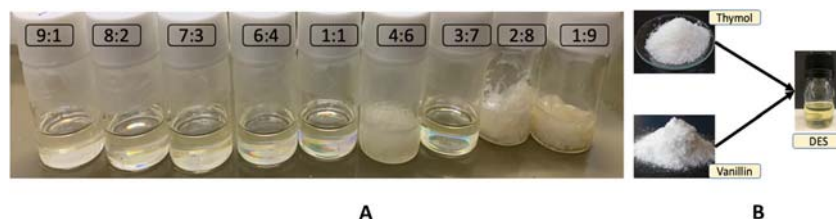


Fig. 3 Preparation of Natural Deep Eutectic Solvent (NADES); (A) different molar ratios of thymol : vanillin and (B) optimised NADES.

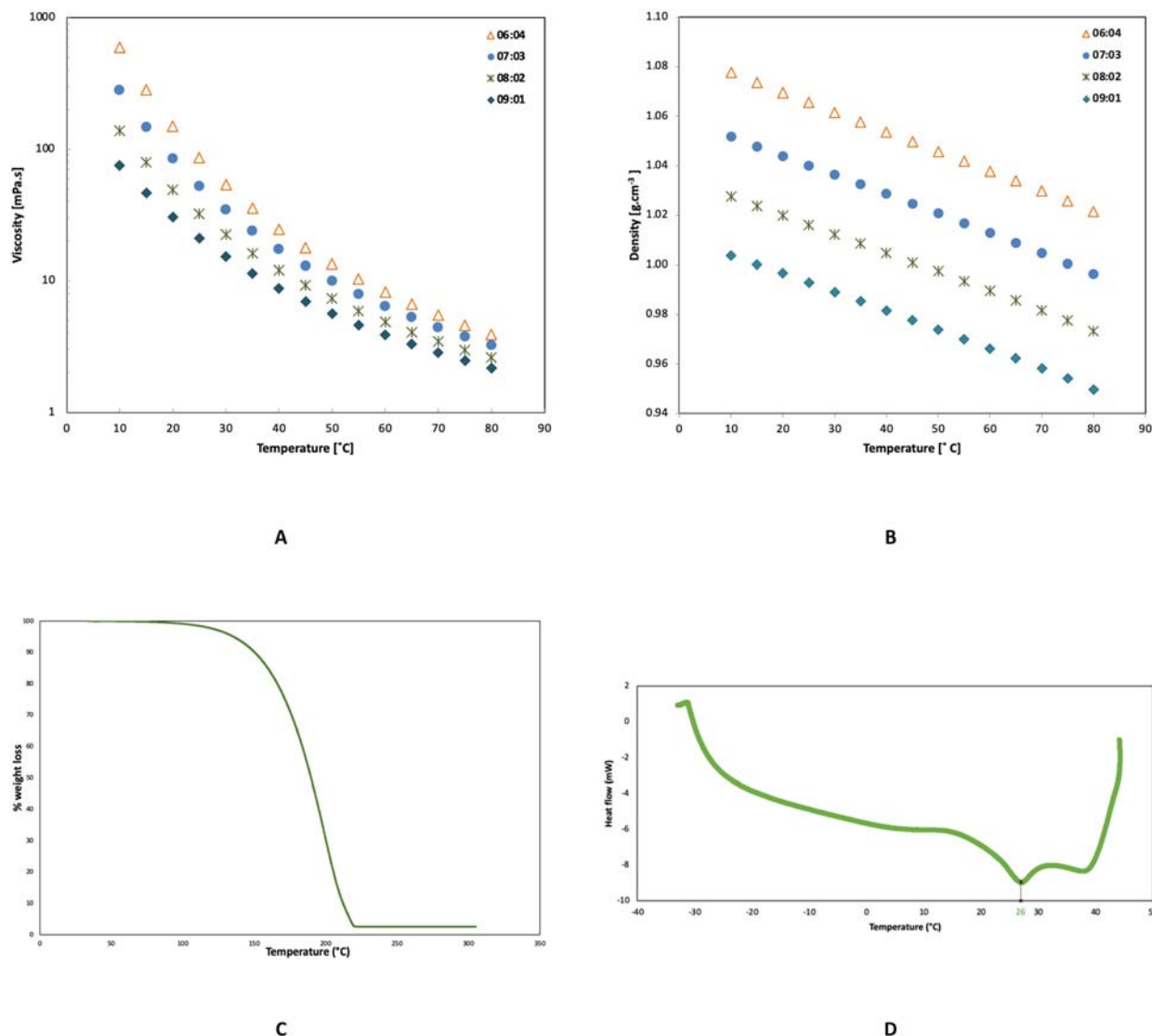


Fig. 4 Physical properties of selected NADES: (A) variation of viscosity with temperature (semi-log plot); (B) variation of density with temperature; (C) thermal degradation properties of the optimised NADES; (D) Determination of melting point of optimised NADES.

Table 1 Solubility of pure PHA in the NADES at different temperatures

Temperature (°C)	30	40	50	60	75
Maximum PHA (% w/w)	5	10	20	35	40
Observation	Clear solution	Clear but viscous	Clear but very viscous	Clear but very viscous	Insoluble

As this downstream process is compared to the ones using chloroform, pure PHA solubility in chloroform was also tested using similar strategy. At first 1% (w/v) PHA was dissolved in chloroform at 30 °C. A quick solubilization was observed. Consequently, we were able to increase the PHA concentration successfully up to 2.5% (w/v), after which it was insoluble. Following the established protocol, the temperature was increased progressively up to 60 °C, and we were not able to

monitor the PHA solution clearness due to the high viscosity of the solution. Thus, it can be concluded that NADES exhibited higher solubility of PHA at any given temperature up to 60 °C.

Characterization of PHA-containing biomass

Given that the recovery yield of PHA extraction can only be calculated based on the PHA content of the starting material it was necessary to analyse the PHA content of the initial lyophilised biomass. By GC-FID analysis, it was found that the biomass contained $48.8 \pm 3.4\%$ of PHA and a3HB : 3HV monomeric content of $84.2 \pm 0.3 : 15.8 \pm 0.3 \text{ wt\%}$.

Downstream processing with NADES and chloroform

PHA extraction methods with chloroform and NADES. With the knowledge of the maximum solubility of pure PHA in chloro-

form, 5% (w/v) PHA, the extraction of PHA from the pre-treated lyophilised biomass was performed as mentioned in the materials and methods section.

The maximum efficiency in terms of purity, recovery and process cost (higher process temperature increases the operational cost) was attained when 10% (w/w) lyophilised powder (corresponding to maximum solubility of 5% (w/w) of pure PHA in NADES; and PHA content in lyophilised powder was ~50%) was added to NADES and stirred at 37 °C for 48 hours. The resultant mixture was filtered with a grade 4 coffee filter.

PHA purification methods (with ethanol after chloroform extraction and with several solvents after NADES extraction). The filtrate with chloroform was purified as per protocol mentioned in materials and methods section.

In the case of filtrate with NADES, the purification step was rather complicated as simple evaporation couldn't be used. NADES is soluble in ethanol and acetone, while PHA is not. However, adding ethanol/acetone to the filtrate (PHA dissolved

in NADES) didn't allow PHA to precipitate. This is because when ethanol or acetone was added to the filtrate containing PHA, both evaporated much faster than the NADES and hence PHA couldn't be purified. To promote the contact time of the filtrate and purification solvent, cold ethanol/acetone (kept in refrigeration at -4 °C for 24 hours) was used. But that also resulted in similar futility.

Thus, less volatile green solvents like isoamyl alcohol and 1-heptanol were tested for purification of PHA. 10x volumes of solvent per volume of filtrate (PHA dissolved in NADES) was added and stirred. The resultant mixture was filtered as mentioned in the "procedures" section. From Fig. 5, it can be clearly observed that 1-heptanol readily separated the PHA from the NADES. With isoamyl alcohol, although PHA was separated from NADES, the recovery efficiency was much lower comparatively, as seen in Fig. 5A and B. Hence 1-heptanol was chosen as the optimised purification solvent. Fig. 6 depicts the purified PHA after drying.

Influence of the extraction step, of the extraction-purification steps and of the ultrasounds on the resultant PHA. Table 2 characterizes different PHA samples, after having different downstream processing steps, in terms of the PHA Purity (%) and PHA Recovery (wt%).

When the extraction of PHA with NADES was applied to the PHA-containing biomass without any pre-treatment, purity values between 10.8% and 70.6% were obtained, indicating the non-reproducibility of this extraction method. To overcome this inconsistency, an ultrasounds step was added to the method, prior to the extraction with NADES, having achieved purity values of *ca.* 98% (Table 3). Thus, it is noteworthy to mention that it was necessary to sonicate the biomass especially when extracted with NADES whereas with chloroform it did not yield much difference. This may be attributed to the fact that chloroform, being a chlorinated polar solvent,^{24–26} is able to disrupt the cell wall for the release of PHA. Whereas in case of NADES, ultrasonic waves disrupt the cell wall and facilitates the consistent release of PHA. The process becomes more energy intensive and physically disruptive with the increase in the amplitude of the sonication waves. Thus 50% ultrasound amplitude was selected for optimum PHA recovery and purity.

Determination of molecular weights using gel permeation chromatography. Molecular weight of PHAs extracted by different downstream processes were determined using Gel

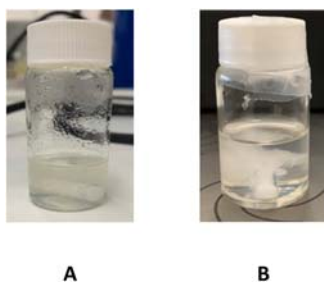


Fig. 5 Purification of PHA enriched NADES by (A) iso-amyl alcohol and (B) 1-heptanol.



Fig. 6 Resultant PHA precipitate (dried in an oven at 60 °C for 6 hours).

Table 2 PHA Purity (%) and PHA Recovery (wt %) of the different PHA samples recovered with different downstream processing steps

PHA sample	PHA purity (wt%)		PHA recovery (wt%)	
	Avg.	Std. Dev.	Avg.	Std. Dev.
Without ultrasound; extracted by CHCl ₃ ; not purified	91.70	3.40	67.40	2.00
Without ultrasound; extracted by CHCl ₃ ; purified by ethanol	98.34	0.77	46.91	0.00
With ultrasound (40% amplitude); extracted by CHCl ₃ ; purified with ethanol	98.78	0.07	47.99	0.02
With ultrasound (40% amplitude); extracted by NADES; purified with 1-heptanol	97.30	3.13	26.71	0.70
With ultrasound (50% amplitude); extracted by NADES; purified with 1-heptanol	98.92	2.20	41.44	0.65
With ultrasound (60% amplitude); extracted by NADES; purified with 1-heptanol	91.03	8.36	50.27	3.26

Table 3 Molecular weight (M_w), number average (M_n), Z average (M_z) and polydispersity (PD) of the recovered PHAs under different downstream conditions

PHA sample	Parameters			
	M_z (kDa)	M_n (kDa)	M_w (kDa)	PD
Without ultrasound; extracted by CHCl_3	234.9	163.9	280.8	1.71
With ultrasound; extracted by CHCl_3	239.7	153.1	275.0	1.80
With ultrasound; extracted by NADES and purified by 1-heptanol	200.1	151.4	259.6	1.71
Without ultrasound; extracted by NADES and purified by 1-heptanol	194.3	169.1	299.4	1.77

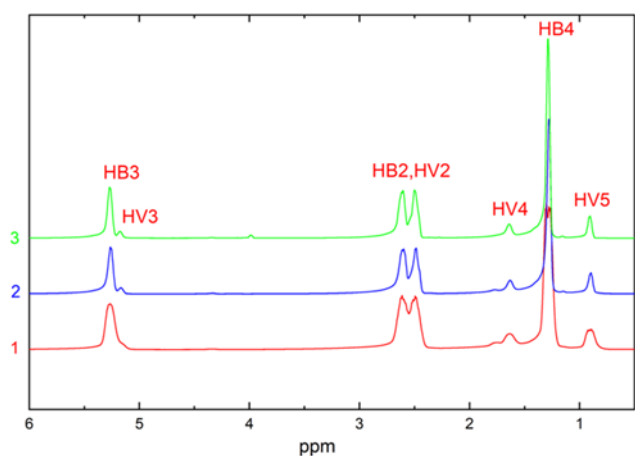
Permeation Chromatography. Several parameters namely M_z (Z average), M_n (number average), M_w (weight average) and PD (poly dispersity) were determined and presented in Table 3.

As observed in the Table 3, sonication has little or no effect on the molecular weights (M_w) when PHA was extracted by CHCl_3 . But M_w is quite different when the extraction was performed with NADES. In presence of ultrasounds, the molecular weight of the purified PHA was smaller in comparison to the one without sonication and CHCl_3 extraction. To confirm that ultrasounds are responsible for the drop in molecular mass, a PHA extracted with NADES without using ultrasounds was characterised. The last row in Table 3 shows that this PHA shows larger M_w , whereas PD is not changed. However, the variation is not huge, thus, it can be safely commented that

the sonication waves didn't alter the polymer quality. Also, the M_w values of the extracted PHA are in agreement with the ones in the existing literature.^{59,66}

NMR characterization. NMR spectra of PHA extracted with the new proposed method are displayed in Fig. 7 together with the spectra of polymers recovered using CHCl_3 . All spectra are qualitatively similar, suggesting that both extraction methods and purifications lead to the recovery of PHBV copolymers showing all proton NMR peaks assigned to HB and HV chemical groups. A shoulder at 1.77 ppm next to the HV2 peak is seen for PHAs recovered with CHCl_3 , which is not present for the other polymer extracted with NADES. A similar peak was noted in the H NMR spectra of MMC PHA recovered with a proprietary extraction route.⁶⁷ The spectrum for the PHA extracted using NADES and purified in 1-heptanol exhibit a peak at 3.97 which is not present in the spectra of polymers extracted with CHCl_3 . Nonetheless, and leaving aside these non-assigned signals, spectra allow for the computation of the ratios of the integrated intensities of HV1 signals over HB1 signals at 0.914 ppm and 1.245 ppm respectively, which give the HV content (in mol %) of each polymer reported in Table 4. HV contents are around 35 mol% HV for these samples. Considering that spectra were acquired with similar resolution, the nearly constant HV content within samples listed in Table 4 suggests that neither the extraction method nor the use of ultrasound significantly affect the chemical structure of the recovered PHBV.

The carbon NMR spectra of extracted PHA are displayed in Fig. 8, which confirms the chemical structure of the recovered biopolyesters with HB and HV chemical groups. To bypass the line broadening in proton NMR spectra shown in Fig. 7, the peaks assigned to HB4 and HV5 between 169 and 169.6 ppm in the carbon NMR spectra can be used to compute the HV ratios in the corresponding polymers as described by Bossu

**Fig. 7** ^1H NMR spectra of samples 1. PHA extracted and purified with CHCl_3 (with ULS); 2. PHA extracted and purified with CHCl_3 (no ULS); 3. PHA extracted with NADES (with ULS), purified by 1-heptanol.**Table 4** Chemical composition of PHA computed from the analysis of NMR spectra

PHA samples	HV content (mol%)				<i>D</i>	
	H NMR		C NMR			
	Avg.	Std. Dev.	Avg.	Std. Dev.	Avg.	Std. Dev.
With ultrasound; extracted with CHCl ₃	34.3	4.6	35.00	2.00	3.30	0.20
Without ultrasound; extracted with CHCl ₃	35.1	3.5	36.50	0.50	3.60	0.80
With ultrasound; extracted by NADES and purified by 1-heptanol	36.9	4.5	48.50	2.50	3.80	0.20

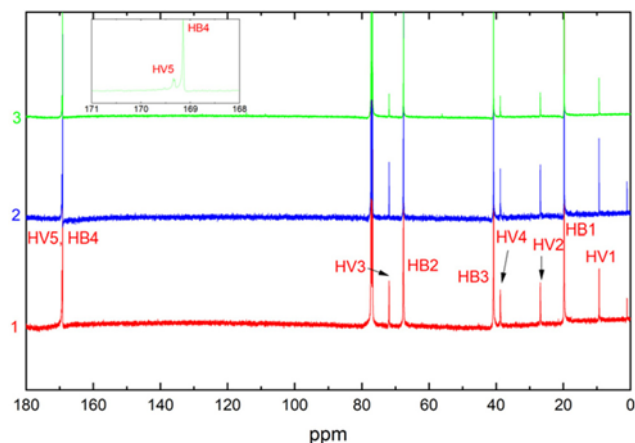


Fig. 8 ^{13}C NMR spectra of samples 1. PHA extracted and purified with CHCl_3 (with ULS); 2. PHA extracted and purified with CHCl_3 (no ULS); 3. PHA extracted with NADES (with ULS), purified by 1-heptanol. Inset: zoom in the HV5 and HB4 peaks for the PHA extracted.

*et al.*⁶⁷ The latter are listed in Table 4 and overall confirm that the use of ultrasonic treatment of the biomass does not impact on the chemical structure of the extracted PHA with CHCl_3 . In contrast to this, the quantitative analysis of carbon NMR spectra suggests that extraction with NADES is more sensitive towards the recovery of PHA richer in HV monomers. However, further PHA characterization and extraction studies with NADES will be needed to clarify this possible selective PHA extraction.

The carbon NMR peaks of HV5 and HB4 groups were also used to compute the D parameter, which gives an indication of the blockiness or the random copolymer nature of the extracted PHBV.^{68,69} All computed D parameters are significantly larger than 1 (see Table 4) and are different from the D parameters computed with the HB1 and HV1 signals of proton NMR should and assuming a Bernoullian random statistics for HB and HV unit along the chain. This result indicates that all recovered PHBV are block copolymers, which is in harmony with the block copolymer nature of other MMC PHA produced in earlier studies.⁶⁷

Recycle/reuse of organic solvents. As mentioned before, a protocol was developed to recycle/reuse the organic solvents. The final filtrate after PHA purification contained $\sim 10\%$ (v/v) NADES and 90% (v/v) 1-heptanol. The separation of the NADES from the 1-heptanol is not an economically viable option as it requires high temperature processes, due to the high boiling temperature of 1-heptanol. Also, it was experimentally observed that this filtrate can be recycled for purification of PHA as long as the percentage of NADES in the filtrate is $<20\%$ (v/v). Hence the first filtrate from the purification step (containing DES and 1-heptanol) can be recycled once again for the purification of PHA.

Thereafter, the second filtrate of the purification step can be probably used both in flavour and fragrance industries. Since NADES contains aroma-based compounds (thymol & vanillin) and 1-heptanol is an odour-active compound, this mixture of solvents can be used as a synthesis intermediate in the fragrance and flavour industries, as it is or diluted in 1-heptanol as per end requirement.^{70,71} A schematic representation of the recycle/reuse protocol is shown in Fig. 9. In doing so, all the organic solvents used in the proposed downstream process were either recycled or reused, one of the essential conditions of 12 principles of green chemistry.⁷²

Conclusions

A new green downstream process was developed for extraction and purification of PHA, a bioplastic. A novel hydrophobic NADES was formulated combining thymol with vanillin in the molar ratio of 8:2, which was characterised in detail. Thereafter the selected NADES system was used to sustainably extract PHA from mixed microbial cultures (MMCs). A better extraction in terms of recovery was attained compared to traditionally used solvents (CHCl_3 in this work). To further purify the PHA, 1-heptanol was the optimised purification solvent. When using NADES as an extraction solvent, a higher PHA recovery and purity was obtained incorporating an ultrasounds pre-treatment step. Using optimised ultrasound amplitude (50%), $\sim 42\%$ PHA was recovered with purity of 99%. The mole-

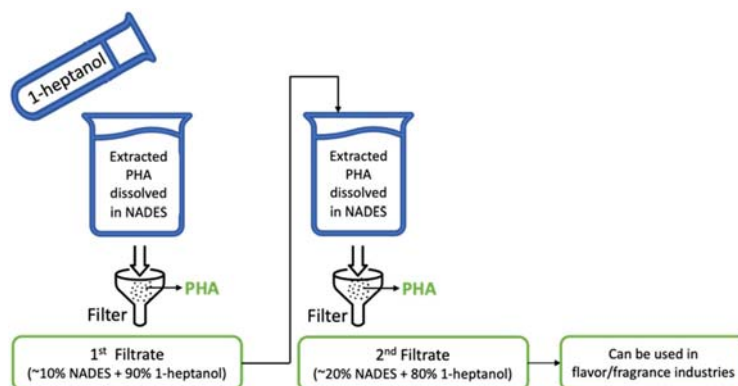


Fig. 9 Proposed recycle/reuse protocol for organic solvents.

cular mass (M_w) of PHA extracted with the NADES protocol was slightly smaller than the PHA recovered with the benchmark protocol. This small reduction is attributed to the ultra-sonication of the biomass needed to assist the NADES-based extraction. The PHA chemical structure assessed by proton NMR does not depend on the down streaming process used in this study. However, further studies will be needed to check the possible selectivity of the NADES-based extraction and purification with 1-heptanol towards PHA with larger HV contents.

Finally, the organic solvents used in extraction and purification, NADES and 1-heptanol respectively, were either recycled into the process or reused in flavours/fragrances industrial applications. The proposed process was compared to a benchmark for PHA downstream, using hazardous chloroform as solvent. We were able to successfully enhance the purity of extracted PHA by 7.5%.

The downstream process proposed in this work is according to the 12 principles of green chemistry, green solvent (NADES) was designed from renewable bioresources (thymol and vanillin); components of NADES exhibited no environmental toxicity;⁷³ harsher solvents (CHCl_3) were replaced with green ones (NADES); safe choice of auxiliary solvent (1-heptanol) was made for purification; the organic solvents used were recycled or reused; no unnecessary by-products were generated; mild processing conditions were used throughout the downstream process ensured high energy efficiency; because of higher efficiency of NADES, less material was used to achieve enhanced recovery of PHA.

The proposed downstream process is a proof-of-concept for extraction and purification of PHAs using sustainable green solvents. Different NADESs can be synthesized to explore the possibility of extraction of different PHAs (mcl-PHAs, homopolymer PHB *etc.*). Also, further optimizations of the processes, such as using wet biomass, can be explored. This sets the premises for future works that can be conducted to investigate further on extraction of PHAs using green solvents.

Author contributions

Conceptualization by A.F.D., S.M., U.T.S., C.B. and M.A.M.R.; investigation, methodology, validation and formal analysis by S.M., U.T.S., C.G. and L.H.; writing – original draft was done by S.M., C.G. and L.H.; writing – review & editing was done by U.T.S., L.H., A.F.D., C.B. and M.A.M.R; work supervision by A. F.D. and C.B.; project administration by A.F.D.; funding acquisition by A.F.D. and M.A.M.R. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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