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Tenebrio molitor-Derived Enzyme Systems Enable Solvent-Reduced Recovery of Intracellular Polyhydroxyalkanoates

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are intracellular microbial polyesters whose commercial deployment is strongly influenced by downstream processing costs and environmental burden. Here, we demonstrate a nearly solvent-free, mild aqueous strategy for the recovery of PHAs directly from wet bacterial biomass using crude enzyme systems derived from *Tenebrio molitor*. Under optimized conditions (0.6 wt% crude protein, pH 7.6, 40°C), near-quantitative (≥95%) recovery of PHB and PHB/HV and up to 60% recovery of mcl-PHA were achieved without prior biomass drying. Proteomic analysis identified abundant digestive hydrolases, including α-amylase and cathepsin and recombinant validation confirmed their contribution to polymer release, demonstrating that targeted enzyme combinations can substantially enhance mcl-PHA recovery (up to 94%–98%). Gel permeation chromatography, NMR and thermal analysis demonstrated preservation of polymer molecular integrity and crystallinity comparable to chloroform extraction. Life cycle assessment revealed a three- to seven-fold reduction in carbon footprint relative to conventional solvent and classical enzymatic methods, primarily due to elimination of drying and solvent use. These findings establish biologically driven biomass hydrolysis as a scalable downstream strategy and highlight insect-derived enzyme systems as promising tools for integrated microbial biopolymer processing.

1 | Introduction

Polyhydroxyalkanoates (PHA) are a well-known class of biopolymers whose industrial applications are increasing due to their unique mechanical properties and biodegradability (Jayalath and Alwis 2025). Once called the emerging materials, they are today already established in specialized sectors such as medical devices, textiles, agriculture and cosmetics (Rodriguez-Contreras 2019; Guzik et al. 2020; Pulingam et al. 2022). The recyclability of these biopolymers has also been explored to integrate them more efficiently into circular economy pathways (Vu et al. 2020; Clarke et al. 2024). Based on the hydroxyalkanoic acid monomer units, PHAs are classified into short-chain length PHAs (scl-PHAs), medium-chain length PHAs (mcl-PHAs) and long-chain length PHAs (lcl-PHAs) (Steinbüchel and Valentin 1995). PHAs are intracellular microbial polyesters accumulated as carbon storage granules that can constitute up to 80% of cell dry weight (Zhang et al. 2024). Many important

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roles have been proposed for these biomolecules including stress protection (Jendrossek and Pfeiffer 2014; Sedlacek et al. 2019). Batch and fed-batch processes are widely used for PHB and PHB/HV production due to their control over nutrient limitation and cell density, whereas continuous cultivation enables finer regulation of polymer composition and is particularly suitable for mcl-PHA synthesis (Koller 2018; Saravanan et al. 2022).

Industrial-scale PHA production (more than 1000 L) is now established at several global producers while pilot operations (100 to 1000 L) have been demonstrated more often using variety of carbon sources (Kacanski et al. 2023; Du et al. 2025; Otero-Logilde et al. 2025). PHAs currently account for 4.1% of bio-based polymer production capacities, which is projected to increase to 17.0% by 2029, making a sustainable and economical production process essential (Bioplastics 2024). The total cost of PHA production comprises raw materials (carbon sources and nutrients used to feed the microorganisms), cultivation and downstream processes such as extraction and purification (Koller 2020; Saavedra del Oso et al. 2021; Thiele and Riedel 2025). Among other challenges, high production costs associated with industrial-scale development still limit PHA's commercialization (Serrano-Aguirre and Prieto 2024). Current research is directed toward the optimization of cultivation strategies and the 'greening' of the extraction protocols Special emphasis is placed on the use of renewable carbon sources including volatile fatty acids and food waste (Du et al. 2025; Khatun et al. 2025) and improvement of the extraction and processing procedures (Mondal et al. 2023; Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025). Although cultivation strategies for PHA production are increasingly optimized, cell harvesting and their further recovery remain among the most energy- and resource-consuming steps, often requiring centrifugation combined with drying of the biomass prior to solvent-based extraction. These operations dominate the energy footprint of PHA production and limit the environmental benefits of otherwise bio-based polymers.

Downstream processing, particularly PHA recovery and purification, remains one of the least explored stages of the PHA production chain despite contributing substantially to overall production costs (Jiang et al. 2018; Koller 2020; Pagliano et al. 2021; Thiele and Riedel 2025). Life cycle assessments (LCA) indicate that these steps dominate the energy demand of PHA manufacturing, often offsetting the environmental advantages of these otherwise bio-based and biodegradable polymers (Saavedra del Oso et al. 2021). Most abundantly utilized solvent-based extraction typically requires carefully dried bacterial biomass, as residual moisture can interfere with polymer solubility, reduce extraction efficiency and promote emulsion formation during contact with the solvent. Dimethyl carbonate, 1,3-dioxolane, deep eutectic solvents and other more sustainable alternatives have been explored instead of chlorinated solvents (Samorì et al. 2015; Yabueng and Napathorn 2018; Didion et al. 2024). Sodium hydroxide (NaOH) has also been explored for sustainable scl-PHA extraction, but with risk of affecting PHA properties (Anis et al. 2012; Rodrigues et al. 2022). Up to 35% reduction in molecular weight of PHB and PHB/HV when exposed to 0.3 M NaOH for more than 4 h was detected (Anis et al. 2013). Moreover, NaOH-based protocols often yield polymers with increased polydispersity indexes (PDI > 2.5), suggesting uncontrolled degradation (Pagliano et al. 2021).

Alternative extraction strategies, including mechanical disruption and cell autolysis approaches, have been explored but present significant limitations (Martínez et al. 2011; Hajnal et al. 2016; Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025). Mechanical methods such as bead milling avoid chemical solvents but often require dried biomass, involve long processing times and remain difficult to scale efficiently (Gutt et al. 2016). More recently, mechanical extraction directly from wet biomass has been reported (Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025) however, despite high recovery yields, the process relies on energy-intensive operations such as repeated high-pressure homogenization and biomass pretreatment. In contrast, enzymatic PHA extraction offers higher selectivity and milder operating conditions. Enzymes such as lipases, proteases and cellulases can selectively degrade cellular components and release PHA granules while preserving polymer integrity (Gonzalez et al. 2021; Pagliano et al. 2021). However, current enzymatic strategies often require relatively high enzyme loadings and are frequently combined with mechanical disruption, chemical treatments or surfactants to achieve satisfactory recovery and purity (Colombo et al. 2020).

A notable biological PHA recovery strategy involves the use of mealworm (*Tenebrio molitor*) larvae, which selectively digest cellular material while excreting intact PHA granules (Murugan et al. 2016). In this process, larvae efficiently consume lyophilized *Cupriavidus necator* biomass and produce faecal pellets containing PHA particles that retain molecular weight and polydispersity comparable to polymers recovered by $CHCl_3$ or NaOH extraction. The approach avoids harsh solvents and enables valorization of residual biomass as feed for mealworms or aquaculture species such as *Oreochromis* sp. (Zainab-L et al. 2022; Shah et al. 2024). Given that mealworms can consume only up to ~10 wt% of their body weight in dried bacterial biomass per day, processing typically requires extended feeding periods of up to 16 days (Zainab-L et al. 2022) or large numbers of larvae, and still necessitates additional purification steps to obtain highly pure polymer fractions (Murugan et al. 2016).

Inspired by this biological principle, we investigated whether enzyme systems derived from *T. molitor* could be harnessed directly for PHA recovery. In this study, crude protein mixtures from *T. molitor* larvae were evaluated for the recovery of PHAs from wet microbial biomass containing polymers with different monomer compositions. The recovered polymers were characterized and compared with those obtained using conventional extraction methods, while the enzymatic basis of the process was explored through proteomic analysis and recombinant expression of selected enzymes. Together, this study establishes insect-derived enzyme systems as an emerging tool for biologically integrated downstream processing of microbial biopolymers.

2 | Material and Methods

2.1 | Materials

All reagents were acquired from commercial suppliers such as Merck/Sigma-Aldrich, Acros Organics or Thermo Fisher Scientific and used as received without additional purification. The 99.8% pure β-D-1-thiogalactopyranoside (IPTG),

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imidazole, $CHCl_3$ and methanol were sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA). NaCl, NaOH, glycerol, biotin, Na_2HPO_4 and KH_2PO_4 were obtained from Sigma-Aldrich (Munich, Germany). Yeast extract, peptone and dextrose were purchased from BioLife (Milan, Italy).

2.2 | Bacterial Biomass, Enzymes and Mealworms

Cupriavidus necator H16 (DSM 428) was cultivated in a 30 L bioreactor (working volume 20 L, Sartorius Biostat C+, Göttingen, Germany) for intracellular poly (3-hydroxybutyrate) (PHB) accumulation following a published protocol (Loan et al. 2022). As the carbon source, soybean oil was used and supplied in a fed-batch mode. After 60 h of cultivation, the biomass concentration reached 135 g L^{-1}, containing 65 wt% PHB. Similarly, *Zobellella denitrificans* MW1 (Ibrahim Mohammad and Steinbüchel 2009) was cultivated on glycerol for 50 h, yielding 81 g L^{-1} of biomass with 67 wt% PHB. In the case of *Pseudomonas putida* CA-3, mcl-PHA was produced over 36 h using rapeseed fatty acids as the principal feedstock. Under optimized fed-batch conditions the process achieved biomass levels exceeding 130 g L^{-1}, with a polymer content of 49 wt%. Following cultivation, the cultures were harvested using a pilot-scale CEPA Z61 tubular centrifuge operating at 17000 rpm (Guzik et al. 2022). The concentrated biomass was subsequently frozen at −80°C and lyophilized for 72 h. The obtained freeze-dried samples were used for PHB extraction and process optimization studies (Figure 1).

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/HV) was produced by cultivating a mixed microbial consortium (MMC) using fermented cheese whey as feedstock (Carvalheira et al. 2022). The MMC, derived from a microbial population of a wastewater treatment plant (Almada, Portugal), was enriched with PHA-storing bacteria within a 100 L sequencing batch reactor operating under alternate feast and famine cycles (12 h) to create a selective pressure. Production of PHB/HV was performed in a 60 L bioreactor, where selected microorganisms were supplied with pulses of fermented cheese whey until the maximum PHA cell content was achieved, indicated by a minimal or absent dissolved oxygen (DO) response. The cultivation was conducted at room temperature (23°C–25°C), with the pH regulated between 8.0 and 8.5, and DO was continuously monitored. At the end of the cultivation period, biological activity was stopped by adding 4 M sulphuric acid to lower a pH between 2 and 3. The cell pellet, with a PHB/HV content of 50 wt% (Carvalheira et al. 2022), was recovered by centrifugation and subsequently lyophilized (Figure 1).

Bromelain from pineapple stem (5 U/mg, B4882), trypsin from porcine pancreas (1000–2000 BAEA U/mg, T4799), pancreatin from porcine pancreas (4× USP, P1750), cellulase from *Aspergillus niger* (0.8 U/mg, 22,178) and α-amylase from *Bacillus* sp. (130 U/mg) were purchased from Merck (Munich, Germany). Savinase (12 KNPU-S/g) and Liquozyme Supra 2.2X amylase (135 KNU/g) were obtained from Novozymes (Copenhagen, Denmark). All enzymes were received in dried form.

T. molitor larvae stock was obtained from the Faculty of Agriculture, University of Novi Sad and the Institute for Biological Research 'Siniša Stanković', National Institute of the Republic of Serbia, University of Belgrade.

2.3 | Gas Chromatography Analysis for PHA Content and Purity

Cell dry weight (CDW) was determined gravimetrically after the biomass was dried to constant mass. The intracellular mcl-PHA and PHB content and monomeric composition were analysed after acid-catalysed methanolysis of lyophilized biomass (10–15 mg) using a mixture of acidified methanol (15% H_2SO_4) and $CHCl_3$, with methyl benzoate as the internal standard (Juengert et al. 2018). The resulting methyl esters of hydroxyalkanoic acids were quantified by gas chromatography with flame ionization detection (GC-FID; Varian CP-3800) on a Zebron ZB-5 capillary column (30 m × 0.32 mm × 0.50 μm) under the following temperature programme: injector 240°C, detector 250°C, 120°C (5 min), ramp to 130°C at 3°C min^{-1}, then to 250°C at 20°C min^{-1}, held 10 min. Commercially available PHB standard (Sigma-Aldrich 915,092, powder with ≥ 98% purity) has been used as standard. For the MMC culture producing PHB/HV, a slightly modified methanolysis procedure was applied according to the literature (Pereira et al. 2023) using 20% (v/v) H_2SO_4 in methanol and chloroform (1:1 v/v) at 100°C for 4 h. After phase separation and purification, the organic phase was analysed by GC–FID (TRACE 1300, Thermo Scientific, USA) with a Stabilwax column (60 m × 0.53 mm × 1 μm). For structural confirmation, the same methyl ester derivatives obtained from all PHA-containing biomasses (PHB, PHB/HV and mcl-PHA) were analysed by gas chromatography coupled with mass spectrometry (GC–MS; Agilent 7890B GC with Agilent 5977A MSD, Waltham, MA, USA). Separation was performed on a Supelco SPB-5 column (30 m × 0.25 mm × 0.25 μm) with an oven programme of 50°C (1 min), ramped at 20°C min^{-1} to 320°C and held for 1 min. The injector operated in splitless mode, and the MS detector was set to full-scan acquisition over m/z 45–400. Peaks corresponding to methyl esters of carboxylic acids and the internal

FIGURE 1 | Dried bacterial biomass samples used in this study for the extraction of PHAs. (a) *Z. denitrificans* MW1, (b) MMC and (c) *P. putida* CA3.

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standard were identified, and monomer structures were confirmed by NIST library matching (probability > 25%).

2.4 | Energy Dispersive X-Ray Analysis (EDX)

EDX was used to examine bacterial biomass. The analysis employed an INCAx-act LN2-free silicon drift detector with PentaFET Precision and Aztec 4.3 software (Oxford Instruments, UK), connected to a TESCAN Mira3 XMU (20 kV, SE detector). All measurements were performed on dry, powdered bacterial biomass.

2.5 | Recovery of PHAs With Commercial Enzymes

A set of commercial enzymes was applied to dried biomass, namely amylase, bromelain, trypsin, pancreatin, savinase and cellulase. For optimisation studies, all biomass samples were suspended in acidic or slightly alkaline buffers (100 mM sodium citrate pH 4.75 or 50 mM Tris–HCl pH 7.6, respectively) and sonicated 5 times on ice for 10 s at 10 μm amplitude. A single commercial enzyme or a mix of commercial enzymes was added, at a concentration of 2 wt% per biomass. The buffer strengths and pH values were selected according to the suppliers' instructions and each mixture was incubated for 8 h at 50°C. Biopolymers were precipitated in cold methanol (2:1 v/v), air-dried and weighed. For the final assessment of enzymatic reactions, harvested cells were concentrated via centrifugation, and without a drying step, enzyme preparations were added.

2.6 | Preparation of *T. molitor* Crude Protein Mix

The worms were fed wheat bran (commercial food) and they were cultivated at room temperature (22°C–25°C) with an air humidity of 70% and without exposure to light. Mealworms (30–40 individual specimens) at approximately 2 weeks post-hatching were transferred onto fresh bran. Under these conditions, the worms were cultivated for 1–3 weeks while monitoring their growth. Every 2–3 days, they were given a small piece of an apple as a water source. The experiment was terminated when the weight of 10 worms reached approximately 1 g, at which point the worms were processed to obtain a crude protein mix for PHA recovery. For the optimization study, mealworms were transferred to 100% *C. necator* H16 biomass containing PHB, 100% *P. putida* CA-3 biomass containing mcl-PHA, as well as mixed substrates 50% bran and 50% *C. necator* biomass, and 80% bran with 20% *P. putida* CA-3 biomass containing mcl-PHA.

Approximately 1 g of cultivated *T. molitor* larvae (7 worms) was flash-frozen with liquid nitrogen, homogenized and resuspended in 10 mL of 50 mM Tris–HCl buffer pH 7.6. The suspension was sonicated on ice for 2 min at 10 μm amplitude, centrifuged at 14,000 g for 30 min at 4°C, and the supernatant was transferred to a new tube as crude *T. molitor* protein mix (TM mix) used for PHA extraction from the wet bacterial biomass. TM mix was either used immediately or stored at 4°C–8°C for a short term or at −20°C for up to 3 weeks.

2.7 | PHA Recovery With Crude *T. molitor* Protein Mix

The *T. molitor* protein mix obtained from worms cultivated on 100% bran (TM mix control) was applied to the biomasses at final concentrations of 0.2%, 0.6%, 1.2% and 2.4% (wt/wt). The mixtures were incubated for 4 and 8 h at 30°C, 40°C and 50°C either in 50 mM Tris–HCl pH 7.6 or in 100 mM sodium citrate pH 4.75. Biopolymers were precipitated in cold methanol, which was reused several times for the precipitation of the same polymer, air-dried and weighed.

The optimal conditions were applied to the *T. molitor* protein mixes TM mixes 1–3 obtained from worms pre-cultivated on a combination of bran and bacterial biomass containing PHAs, namely 50% bran with 50% *C. necator* biomass (TM mix 1), 100% *C. necator* biomass (TM mix 2) and 80% bran with 20% *P. putida* CA-3 biomass (TM mix 3) were applied to the biomass in final concentrations of 0.2% (wt/wt). The mixtures were incubated for 8 h at 50°C in 50 mM Tris–HCl pH 7.6.

Generally, wet bacterial biomasses from *Z. denitrificans* MW1, MMC and *P. putida* CA-3 were used for the PHA recovery assessment. For the precise determination of the optimum amount of enzyme per bacterial biomass, the dried biomass has been utilized.

2.8 | MMC Population Identification

Fluorescence in situ hybridization (FISH) analysis was carried out on 4% paraformaldehyde-fixed biomass samples to identify the population present in the SBR biomass and was performed as described previously (Amann et al. 1995). The oligonucleotide probes used in FISH were: EUBMIX (for all bacteria), comprising EUB338, EUB338-II and EUB338-III, PAR651 for *Paracoccus*, Azo644 for *Azoarcus*, THAU832 for *Thauera*, LAMP444 for *Lampropedia*, AMAR839 for *Amaricoccus*, Zra23a for *Zoogloea*, UCB-823 for *Plasticicumulans acidivorans*, ULB-450 for *Plasticicumulans lactivorans*, Meg983 and Meg1028 for *Meganema*. Details about each probe can be found at probeBase (Greuter et al. 2016). The biomass samples were observed using an Olympus BX51 epifluorescence microscope (Japan) at 1000 X.

2.9 | Characterization Methods for the Recovered PHAs

2.9.1 | Gel Permeation Chromatography (GPC)

Recovered PHA materials obtained by different downstream processes, using $CHCl_3$, optimal commercial enzyme (α-amylase), *T. molitor* mix (TM control mix) and recombinant α-amylase, were characterized for their molecular weights and polydispersity by GPC (Pereira et al. 2023). The obtained samples (15 mg) were dissolved in 3 mL of $CHCl_3$, at room temperature, and filtered through a filter 47 mm (PTFE). 100 μL of the samples was injected for the analysis by GPC System (Waters Millenium) supported with columns: PLgel 5 μm Guard; Polymer Laboratories; 50 × 7.5 mm, PLgel 5 μm

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104 Å; Polymer Laboratories; 300 × 7.5 mm, PLgel 5 μm 500 Å; Polymer Laboratories; 300 × 7.5 mm. As the mobile phase, $CHCl_3$ was used, at a flow rate of 1.0 mL min^{-1} at 30°C and a RI detector (Waters 2410) was used for polymer detection. By applying monodisperse polystyrene standards with molecular weights ranging between 800 Da and 504 kDa the calibration curve was obtained. The number-average Mn, weight-average Mw and PDIs were determined from these measurements.

2.9.2 | Nuclear Magnetic Resonance (NMR)

Bruker Ascend 400 instrument (Bruker BioSpin GmbH, Germany) was used to record all NMR spectra (^1H NMR at 400 MHz, ^{13}C NMR at 100 MHz). Deuterated chloroform ($CDCl_3$, Acros Organics, Darmstadt, Germany) was used as solvent, and tetramethylsilane (TMS) was used as internal standard. Chemical shifts (δ) are reported in ppm, and coupling constants (J) are given in Hz.

2.9.3 | Differential Scanning Calorimetry Analysis (DSC)

DSC measurements were performed using a Shimadzu—DSC-60 Plus (Shimadzu Corporation, Kyoto, Japan) in an inert atmosphere, nitrogen flow of 50 mL min^{-1}. The analysis were performed in the temperature range from −40°C to 250°C, at a heating rate of 10°C min^{-1}. The testing sample mass was limited to 5.0 ± 0.5 mg. From the recorded thermograms, characteristic parameters were determined: melting temperature, T_m and melting enthalpies, ΔH_m, using OriginPro 9.0 software. The melting enthalpies were determined from the area under the endotherms, while the degree of crystallinity, X_c, was calculated using melting enthalpy, ΔH_m and the melting enthalpy od 100% crystalline PHA, (146 J/g) (Modi et al. 2011) applying the Equation (1):

$$X_c = {}^{\Delta H_m}/_{\Delta H_m^0} \times 100\,(\%) \quad (1)$$

2.10 | Proteomic Study of *T. molitor*

The proteome of *T. molitor* after growth on 100% bran (TM mix control) and on 100% *C. necator* biomass (TM mix 2) was analysed with LC–MS/MS proteomics using three biological replicates and each biological replicates were analysed in three technical replicates. Protein extraction and digestion and LC–MS/MS settings are described in Supporting Text S1 (Hughes et al. 2019). The mass spectrometer operated in data-independent acquisition (DIA) mode. Raw files were searched together using DIA-NN v2.2.0 (Demichev et al. 2020) against the *T. molitor* reference proteome (Refseq accession GCF_963966145) (Mann et al. 2024) filtered to retain the longest isoform per gene, and a proteomics contaminant library (Frankenfield et al. 2022). Relative protein quantification was performed with the QuantUMS algorithm and differential protein abundance was assessed using the LIMMA moderated test statistic with a Benjamini-Hochberg FDR of 5% (Ritchie et al. 2015). Absolute protein abundance was estimated using the intensity-based absolute quantification (iBAQ) algorithm (Silva et al. 2006). Detailed methodology of the data processing is presented in Supporting Text S1.

Proteins in PHA biomass-fed worms, identified through proteomic analysis, were compared to commercially available enzymes previously utilized for PHA extraction, namely bromelain from the pineapple (UniProt P14518), trypsin (UniProt P00761), savinase (UniProt P29600), pancreatic lipase (UniProt P00591), pancreatic α-amylase (UniProt P00690) and endo-β-1,4-glucanase (UniProt Q9L3J2). Homology was assessed using BLAST (Basic Local Alignment Search Tool), applying cutoff criteria of 40% identity and 70% query coverage.

2.11 | Expression and Purification of *T. molitor* α-Amylase and Cathepsin

Codon-optimized genes for α-amylase (P56634) and cathepsin (A0A0B5IPN6) in *T. molitor* were synthesized (GenScript Biotech, Nanjing, China) and cloned into the pPICZαA vector for methanol-inducible secretion in *Pichia pastoris* X-33. Transformation was performed by electroporation following the protocol of the Invitrogen EasySelect Pichia Expression Kit (Thermo Fisher Scientific, Waltham, MA, USA). Transformants were selected on YPD agar supplemented with zeocin (100 μg/mL) and screened for protein expression in BMGY medium following the manufacturer's instructions. For large-scale expression, cultures were grown in BMGY to an OD600 of approximately 2, then transferred to BMMY medium and induced with daily additions of methanol (0.75% v/v for cathepsin; 1.5% v/v for α-amylase) for 5 days at 30°C. Supernatants were obtained by centrifugation and filtration, lyophilized and resuspended in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). Proteins were dialyzed overnight at 4°C using a 10.000 MW membrane and purified under native conditions using Ni-NTA agarose (Qiagen, Hilden, Germany). Purity was assessed by 12.5% SDS-PAGE (Bio-Rad, USA) and proteins were quantified using the BCA1-1KT bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Further experimental details, including colony screening and buffer compositions, are provided in the Supporting Text S2.

α-Amylase activity was evaluated using the clear-zone method on starch agar plates. A 2% starch agar plate was prepared, and different volumes (10–20 μL) of the extracted α-amylase were applied. The plates were incubated at 37°C for 24 to 96 h. After incubation, the agar surface was stained with iodine vapour (4 mg of iodine was sprinkled directly on the empty part of agar plate). The presence of a clear zone around the enzyme application site indicated starch hydrolysis.

2.12 | LCA Inventory Assumptions and Methods

For comparison, all methods were modelled based on the requirement of 2.5 kg of dry bacterial biomass to produce 1 kg of PHA, assuming 50% polymer content in the cells and 80% extraction recovery. The $CHCl_3$ method required complete drying of the wet biomass and the use of halogenated solvents. The classical enzymatic method remained aqueous but relied on methanol for precipitation. The new method with *T. molitor* used

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only 0.6 wt% crude protein as the enzymatic reagent, operated at ~40°C and did not require organic solvents or prior drying of the biomass suspension.

A detailed life cycle inventory (LCI) and individual scenarios are discussed in the accompanying Supporting Information. The impact assessment (LCIA) was carried out based on the simplified carbon footprint method of the Greenhouse Gas Protocol and is expressed in carbon dioxide equivalent.

2.13 | Statistical Analysis

Results are presented as mean ± standard deviation (SD). Statistical analysis of PHB, PHB/HV and mcl-PHA recovery (wt%) was performed using one-way analysis of variance (ANOVA, single factor), with Fisher's least significant difference (LSD) test applied for post hoc comparisons, following significant ANOVA results. Statistical significance was considered at $p \leq 0.05$. All analyses were carried out using the Data Analysis ToolPak in Microsoft Excel. QuantUMS protein intensities were centred and subjected to principal component analysis (PCA) after excluding the 10% of proteins with the lowest variance.

3 | Results

3.1 | PHA- Containing Bacterial Biomass Characterization

In this study, both pure and mixed bacterial cultures were used for the PHA extraction (Figure 1). High-cell-density fed-batch cultivations enabled efficient PHA accumulation in all three model strains. Gram-negative *Z. denitrificans* MW1 grown on glycerol achieved 81 g L^{-1} of biomass containing 67 wt% PHB after 50 h. *P. putida* CA-3 cultivated on rapeseed fatty acids produced 130 g L^{-1} biomass with 49 wt% mcl-PHA within 36 h, consistent with previously optimized fed-batch conditions (Guzik et al. 2022). *C. necator* H16 reached a biomass concentration of 135 g L^{-1} with 65 wt% PHB after 60 h of cultivation on soybean oil and was predominantly used for *T. molitor* feeding experiments. The MMC biomass cultivated on fermented cheese whey as the sole feedstock had a PHB/HV content of 50 wt%, with an HV content of 34%. The MMC was obtained in a sequencing batch reactor operated under feast and famine cycles for the selective enrichment of PHA accumulating organisms. This strategy successfully selected an MMC enriched in PHA-storing organisms, in which members of the genera *Lampropedia, Paracoccus, Azoarcus* and *Thauera* were detected, with Gram-negative non spore-forming coccus *Lampropedia* being the most abundant among the PHA-storing organisms (Figure S1). These observations were in accordance with previous studies, since *Paracoccus, Azoarcus* and *Thauera* are commonly detected in PHA-producing systems using fermented streams as feedstocks.

The EDX spectra of bacterial biomass powders, indicating their elemental composition, are shown in Table 1. Based on the observed results, the most abundant elements were carbon (50–60 wt%), oxygen (27 wt%–42 wt%) and nitrogen (1 wt%–14 wt%), indicative of organic material and bacterial biomass elemental stoichiometry reported previously (Cleveland and Liptzin 2007; Scott et al. 2012). MMC showed significantly lower N and P content by order of magnitude in comparison to two single cultures (Table 1). Other detected elements in relatively small amounts such as Na, Mg, S, K, Fe and Si, were expected to be present in bacterial biomass as they are essential for enzyme function, structural integrity and electron transport. The total amount of biopolymer within bacterial biomass was comparable (49 wt%–67 wt%). Both, wet and freeze-dried biomass samples were subsequently used for biopolymer extraction and comparative analysis of recovery efficiency.

3.2 | Recovery of PHAs With Commercial Enzymes

Six commercial enzymes (amylase, cellulose, bromelain, trypsin, pancreatin and savinase) have been selected for this study

TABLE 1 | Compositional analysis of the bacterial biomasses using EDX analysis.

	Z. denitrificans MW1		Mixed microbial culture (MMC)		*P. putida* CA-3	
Element	**wt %**	**σ, Wt%**	**Wt, %**	**σ, Wt%**	**Wt, %**	**σ, Wt%**
C	53.96	0.61	57.13	0.43	52.90	1.10
N	4.48	0.03	0.89	0.02	13.71	1.44
O	39.28	0.61	40.84	0.44	27.86	0.72
Na	n.d.	n.d.	0.53	0.05	0.70	0.06
Mg	n.d.	n.d.	n.d.	n.d.	0.44	0.04
P	1.08	0.05	0.16	0.02	2.49	0.08
S	0.19	0.04	0.27	0.03	0.88	0.05
K	0.35	0.04	n.d.	n.d.	0.34	0.03
Fe	n.d.	n.d.	n.d.	n.d.	0.68	0.06
Si	n.d.	n.d.	0.18	0.02	n.d.	n.d.

Abbreviation: n.d., not detected.

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(Table 2). In addition, we evaluated the effectiveness of different enzyme combinations in extracting PHAs from the three distinct microbial sources under 2 pH conditions (7.00 or 4.75), for 8 h at 50°C and 2 wt% enzyme load per biomass (Table 2). To benchmark the performance of enzymatic recovery against conventional solvent extraction, dried PHA-containing biomasses were subjected to both $CHCl_3$ and enzymatic treatments (Tables 2 and 3). Solvent extraction with $CHCl_3$ yielded nearly quantitative recoveries of 96.7% for *Z. denitrificans* MW1 PHB, 95.1% for MMC PHB/HV and 99.5% for *P. putida* CA-3 mcL-PHA (Table 3).

The PHA recoveries reached with single enzymes were between 41% and 95% (Table 2). Commercial amylase and protease Savinase were tested for the first time for PHA recovery. Amylase achieved excellent recoveries of up to 86%, whereas savinase was unsuccessful in releasing biopolymers from bacterial biomass, with a maximum recovery of 21% from mcl-PHA in *P. putida* CA3 (Table 2). Combining enzymes generally improved recovery; combinations of amylase and trypsin, and bromelain and trypsin, increased yields by ~10% compared to single enzymes, specifically for *Z. denitrificans* and MMC, suggesting a synergistic effect of proteolysis and polysaccharide degradation. However, more complex enzyme cocktails did not increase recovery and, in some cases, caused a significant decrease, likely due to competitive interactions or excessive degradation of biomass components. The results indicated that proteolytic enzymes offer a promising approach for PHA extraction under mild aqueous conditions.

TABLE 2 | Recovery of PHAs from three bacterial biomasses after various enzyme treatments at 2 wt%.

Enzyme/ Combination	Bacterial species and PHA type		
	Z. denitrificans MW1 (PHB)	MMC (PHB/ HV)	*P. putida* CA-3 (mcl-PHA)
Amylase	83%[a]	85%[a]	86%[a]
Cellulase	80%[b]	65%[b]	80%[b]
Bromelain	62%[b]	52%[a]	41.5%[a]
Trypsin	88%[a]	65%[a]	95.2%[a]
Pancreatin	85%[a]	86.7%[a]	81.6%[a]
Savinase	2.5%[a]	2.5%[a]	21.8%[a]
Amylase-trypsin	90.6%[a]	85%[a]	92.5%[a]
Amylase-bromelain	88.5%[a]	85%[a]	54.4[a]
Bromelain-trypsin	90.8%[a]	14%[a]	1.0%[a]
	90.0%[b]	81.6%[b]	1.4%[b]
Bromelain-trypsin-pancreatin	90%[b]	61.3[b]	90%[b]
Bromelain-trypsin-pancreatin-cellulase	21.1%[a]	5%[a]	9.5%[a]
	20.8%[b]	5%[b]	4.1%[b]

Note: Buffers used.
[a]50 mM Tris–HCl pH 7.6.
[b]100 mM sodium citrate pH 4.75.

TABLE 3 | Extraction efficacy of the crude *T. molitor* protein mix compared to the $CHCl_3$ and commercial enzyme methods.

Bacterial biomass	PHA type	Extraction method	Recovery (%)
Z. denitrificans MW1	PHB	*T. molitor* protein mix	99.9
		α-amylase	83.0
		$CHCl_3$	96.7
MMC	PHB/HV	*T. molitor* protein mix	95.6
		α-amylase	85.0
		$CHCl_3$	95.1
P. putida CA-3	mcl-PHA	*T. molitor* protein mix	59.8
		α-amylase	86.0
		$CHCl_3$	99.5

For PHB, proteolytic enzymes proved most effective. Trypsin achieved the highest recovery (88%), closely followed by pancreatin (85%) and amylase (83%). Synergistic effects were observed in double-enzyme combinations, with amylase-trypsin and bromelain-trypsin exceeding 90% recovery. The MMC PHB/HV system showed more variable results. Amylase, pancreatin and amylase-trypsin reached ~85% recovery, while bromelain alone was less effective (52%). Trypsin alone yielded moderate recovery at 65%, suggesting that the more complex biomass matrix in MMC requires customized enzyme combinations rather than a single protease. The bromelain-trypsin combination improved recovery to ~90% (Table 2), demonstrating that specific enzyme synergies can enhance MMC lysis. For the least studied mcl-PHA, trypsin provided the highest recovery (95.2%), followed by amylase (86%) and pancreatin (81.6%) (Table 2). The combination of amylase and trypsin maintained high efficiency (92.5%), suggesting that customized enzyme cocktails can enable selective extraction of mcl-PHA from Gram-negative bacteria under mild conditions with high yield.

3.3 | Use of *T. molitor* Crude Protein Mixes for PHA Recovery

The crude *T. molitor* protein mix obtained from mealworms cultivated on bran (TM mix control) was employed for a successful PHAs extraction from three different bacterial biomasses under different conditions (Figure 2). The 50 mM Tris–HCl buffer pH 7.6 was selected, as it was the preferred medium for commercial enzymes (Table 2). Usually, from 1 g of mealworms (seven worms; 130–150 mg per worm), the yield of crude protein mix was 8 mL containing 3.5 mg/mL of protein. Under these optimized aqueous conditions, the crude *T. molitor* protein mix exhibited remarkable extraction efficiency confirming that spectra

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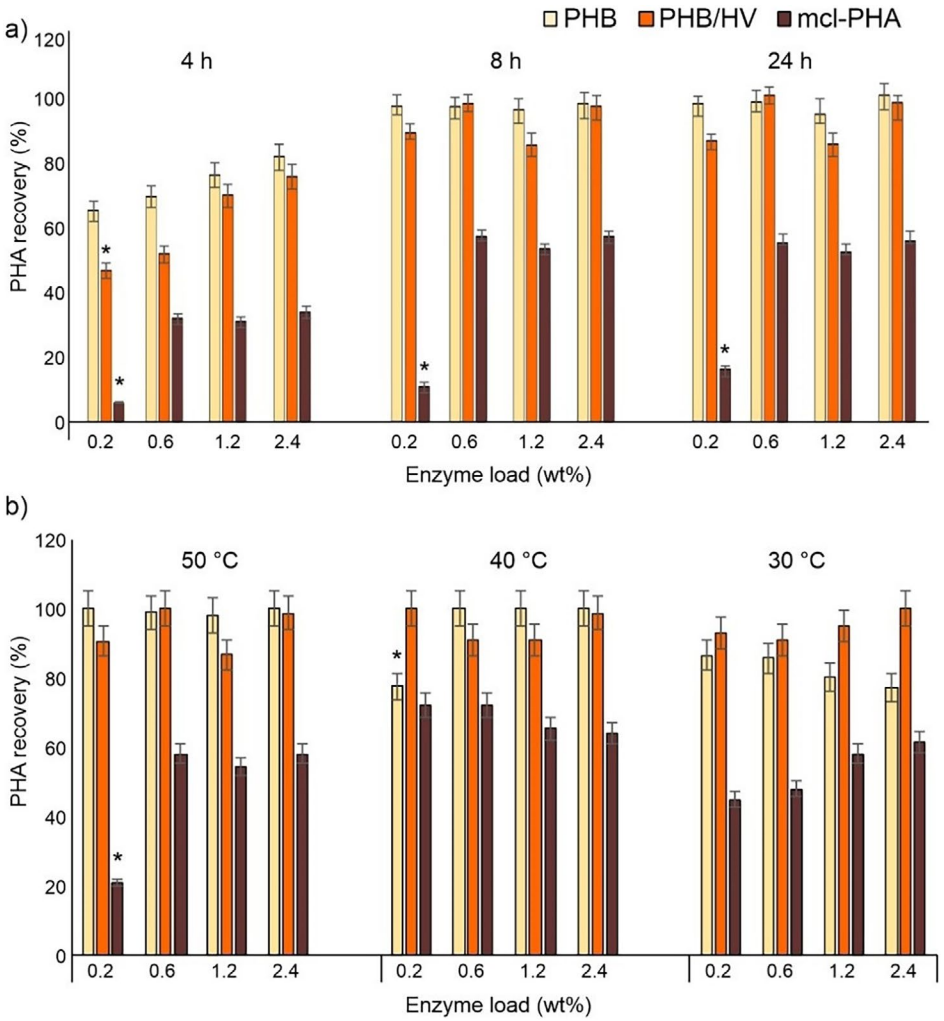

FIGURE 2 | Optimization of PHB, PHB/HV and mcl-PHA recovery (wt%) using crude *T. molitor* protein mix. (a) Different enzyme loads (0.2 wt%–2.4 wt%) were tested at 50°C in 50 mM Tris–HCl buffer pH 7.6 for 4, 8 or 24 h. (b) Temperature optimization with 0.2 wt%–2.4 wt% of enzyme mix employed for 8 h at 50°C, 40°C and 30°C. Statistical analysis (ANOVA test and post hoc Fisher's LSD test; $*p \leq 0.05$ was considered statistically significant) was performed for each biopolymer at each of the tested conditions.

of different groups of digestive enzymes are secreted and simultaneously operating (Table 3).

For *Z. denitrificans* MW1, PHB recovery reached almost 100%, outperforming the conventional $CHCl_3$ and commercial enzymatic methods. Similarly, recovery rates of PHB/HV from MMC also yielded > 95%, closely matching solvent extraction (95.1%) and exceeded recovery by the commercial α-amylase by 1.1-fold (Table 3). In contrast, mcl-PHA from *P. putida* CA-3 biomass recovery was approximately 60%, which was both lower than solvent extraction and α-amylase treatment by 1.6 and 1.4-fold, respectively. However, this was much higher than recoveries obtained by single enzymes such as bromelain or savinase (Table 2). These findings demonstrate that the naturally balanced enzymatic composition of the *T. molitor* extract, containing proteases, glycosidases and other hydrolases, provides synergistic activity capable of degrading cell envelopes and liberating intact PHA granules on a range of biomass and PHA types.

The protein load was assessed between 0.2 wt% and 2.4 wt% per biomass, and the incubation times of 4, 8 and 24 h were examined (Figure 2a). For PHB and PHB/HV, the TM control mix load did not play a major role, as satisfactory enzymatic activity had already been reached at 0.2 wt%. For mcl-PHA, the lowest load affords < 15% in all time points, but concentrations between 0.6 wt% and 2.4 wt% gave recovery yields of 40%–60% (Figure 2a). In comparison to 2 wt% loads of commercial enzymes (Table 2), utilizing TM control mix enables higher recovery rates at 10 times lower protein load. The optimal incubation time was 8 h, as shorter time (4 h) showed incomplete recovery, while the prolonged exposure (24 h) resulted in no significant PHAs yield improvement (Figure 2a). To further lower the energy demand for PHAs extraction, recovery experiments were carried out at 40°C and 30°C (Figure 2b). Surprisingly, lowering the temperature to 40°C resulted in comparable and slightly higher recovery rates for all PHA types, especially mcl-PHA where values of up to 72% were achieved (Figure 2b). Further lowering of the extraction temperature resulted in slightly lower yields.

3.4 | GPC and NMR of Recovered PHAs

Representative images of recovered biopolymers using TM protein mix without any further purification steps are shown

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in Figure 3. Mcl-PHA appeared slightly yellowish in colour, which may be due to strain and fermentation conditions, but also to co-extracted pigments, such as flavins, carotenoids and phenazines known to be produced by *Pseudomonas* spp. (Dartiailh et al. 2020; Lu et al. 2024).

GPC was used to determine the molecular weights of PHAs extracted using different downstream processes (Table 4). The GPC curves of all PHAs were unimodal (data not shown), and the molecular weight distribution was relatively narrow, varying with the selected downstream process. In the case of PHB extraction, TM control mix slightly enhanced polymer recovery by preserving longer chains due to the highest M_n (97.9 kDa) observed compared to the standard solvent method using chloroform (89.1 kDa). Using α-amylase across recovery yielded comparable molecular weights to those measured using chloroform. A similar trend was followed for the M_w values, while the PI values increased using the TM control mix recovery method, due to the higher M_w and M_n values indicating broader chain length distributions. The different enzymatic components available in the crude protein mix may reflect less selective recovery and a higher PI. However, the obtained results indicated that applying TM crude protein mix resulted in good quality of the extracted PHB. When the PHB/HV biopolymer was extracted using the TM control mix, approximately two-times lower molecular weight was observed (M_n 42.9 kDa and M_w 91.7 kDa) compared with chloroform extraction, with a slightly higher PI, similar to that observed for PHB. In the recovery of PHB/HV, α-amylase appeared highly efficient, yielding high molecular weights, suggesting selectivity and targeting specific components of the microbial cell wall (e.g., polysaccharides or proteins) while keeping the intracellular PHA granules intact. All three applied recovery methods in the case of mcl-PHA resulted in the low molecular weights consistent with its nature. The PHAs recovered by different approaches were further characterized by NMR (Figures S2–S5). In all three ^{1}H NMR spectra, characteristic signals were observed: methyl protons at 1.26–1.28 ppm (1), methylene protons at 2.44–2.63 ppm (2) and methine protons at 5.22–5.30 ppm (3). Complementary ^{13}C NMR spectra (Figure S3) further corroborated the presence of the expected four peaks: methyl (CH_3) at 19.90 ppm, methylene (CH_2) at 40.94 ppm, methane (CH) at 67.76 ppm and carbonyl (C=O) at 169.28 ppm, reinforcing the conclusion that the TM protein mix yields PHB with an intact and well-defined chemical structure. All spectral properties were in line with the literature (Jiang et al. 2018) confirming the undamaged chemical structure and purity of samples obtained by the TM protein mix.

FIGURE 3 | Photographs of different PHAs recovered by crude *T. molitor* protein mix.

DSC analysis provided the information about thermal profiles of recovered PHAs. In the thermograms of extracted PHB, a single endothermic melting peak was observed for all samples, with melting temperatures (T_m) ranging from 171.4°C to 174.2°C (Table 4, Figure S6) which is in correlation with the expected values for this type of polymer (Mondol et al. 2025). While the T_m of PHB recovered using different methods was quite similar, the corresponding melting enthalpies (ΔH_m) notably varied, additionally affecting the polymer crystallinity. Hence, the PHB recovered using TM control mix indicated high ΔH_m and degree of crystallinity of 37.8%, which was higher compared to the PHB recovered by chloroform, while the PHB recovered by α-amylase was indicated as a material of low crystallinity (3%), stressing the influence of the selected recovery method on thermal properties of PHB. Similarly, thermal properties of the PHB/HV polymer followed a comparable trend in terms of influence of recovery method on characteristic thermal parameters—TM control mix recovered material of better thermal properties in comparison to the one extracted using $CHCl_3$ as solvent. The first endothermic melting peak (T_{m1}) observed at 68°C to 72°C is attributed to the 3-hydroxyvalerate (HV) fraction, whereas the second, higher-temperature peak (T_{m2}), which ranged

TABLE 4 | Number-average (M_n), molecular weight average (M_w) and polydispersity index (PDI), melting temperature (T_m), melting enthalpy (ΔH_m), degree of crystallinity (X_c) of the recovered PHB, PHB/HV and mcl-PHA using various recovery methods, followed by thermal analysis.

PHA type	Recovery method	M_n (kDa)[a]	M_w (kDa)[a]	PDI	T_m, °C	ΔH_m, J/g	X_c, %
PHB	TM control mix	97.9	**256.6**	2.62	171.4	55.2	37.8
	α-amylase	85.2	183.3	2.15	173.7	4.40	3.01
	$CHCl_3$	89.1	190.2	2.13	174.2	56.4	36.6
PHB/HV	TM control mix	42.9	91.7	2.14	71.7/144.5	26.1	17.8
	α-amylase	**92.5**	**195.4**	2.11	38.2/138.8	13.7	9.38
	$CHCl_3$	62.8	104.2	1.66	67.8/154.0	27.7	19.0
mcl-PHA	TM control mix	13.3	24.6	1.85	n.d.	n.d.	n.d
	α-amylase	13.1	23.4	1.78	73.8	n.d.	n.d.
	$CHCl_3$	18.5	28.7	1.55	78.6	n.d.	n.d.

Abbreviation: n.d., not detected.
[a]The values represent the average of three independent measurements with a standard deviation between 5% and 10%. Within each group statistically different values are in bold.

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from 140°C to 155°C, is associated with the HB fraction. Contrary to PHV and PHB/HV, the recovered mcl-PHA exhibited melting temperatures of ~78°C, a broad peak due to its flexibility, and very low crystallinity. The mcl-PHA recovered using the TM control mix was of even lower crystallinity and a complete absence of a melting peak was observed, while in the case of α-amylase and chloroform, this peak appeared of low intensity and very broad. Based on determined M_n, PDIand T_m values (Table 4) expected T_g values for PHB, PHB/HV and mcl-PHA are 5°C–10°C, −50°C to −15°C, respectively (Sharma et al. 2017; Nikodinovic et al. 2008).

3.5 | Proteomic Studies of *Tenebrio molitor*

With a view of increasing the efficiency of mealworm protein mix, they were pre-grown on bacterial biomass and substrates containing bacterial biomass. When grown on bacterial biomass only, these worms failed to develop properly, particularly those grown on *P. putida* cells, and they appeared darker in colour (Figure S7). Therefore, bran was added in different proportions to the bacterial biomass to obtain good mealworm growth observed only when 50% of bran was mixed with *C. necator* biomass and when 80% of bran was mixed with 20% of *P. putida* biomass (results not shown). Crude protein mixes from mealworms pre-grown on 50% and 100% *C. necator* biomass were labelled as TM mix 1 and 2, respectively, while the crude protein mix obtained from mealworms pre-grown on 20% *P. putida* biomass was labelled as TM mix 3. TM mixes 1–3 showed comparable or reduced efficiency of the extraction of various PHAs in comparison to the TM control mix (Figure 4a). TM mixes 1–3 showed that the PHB recovery was between 66% and 100%, 93%–96% was observed for PHB/HV, while recovery for mcl-PHA remained lower, between 6% and 58% (Figure 4a). Although pre-growth of *T. molitor* on substrates containing biomass did not yield more efficient PHAs recoveries, SDS-PAGE analysis revealed distinct banding patterns among TM mixes, particularly TM mix 2 (pre-growth on 100% *C. necator* biomass), suggesting differential growth and protein expression associated with microbial biomass (Figure S8). TM mix 2 displayed a thick array of bands, particularly in the 28–63 kDa range, indicating the presence of microbial metabolic and structural proteins. TM mix 1 (pre-growth on a combined 50% bran with 50% *C. necator* biomass) exhibited a hybrid profile.

To identify which enzymes from *T. molitor* are responsible for the efficient PHA recovery and to determine their structure and function in the interaction with the bacterial biomass, a proteomic study was conducted. We analysed the proteome of *T. molitor* after growth with bran (TM control mix) and bacterial biomass (TM mix 2). Each condition included three biological replicates, and each biological replicate was analysed in three technical replicates. Technical replicates were highly similar, clustering together in principal component analysis (PCA) (Figure 4b), and had a Pearson correlation of more than > 0.998.

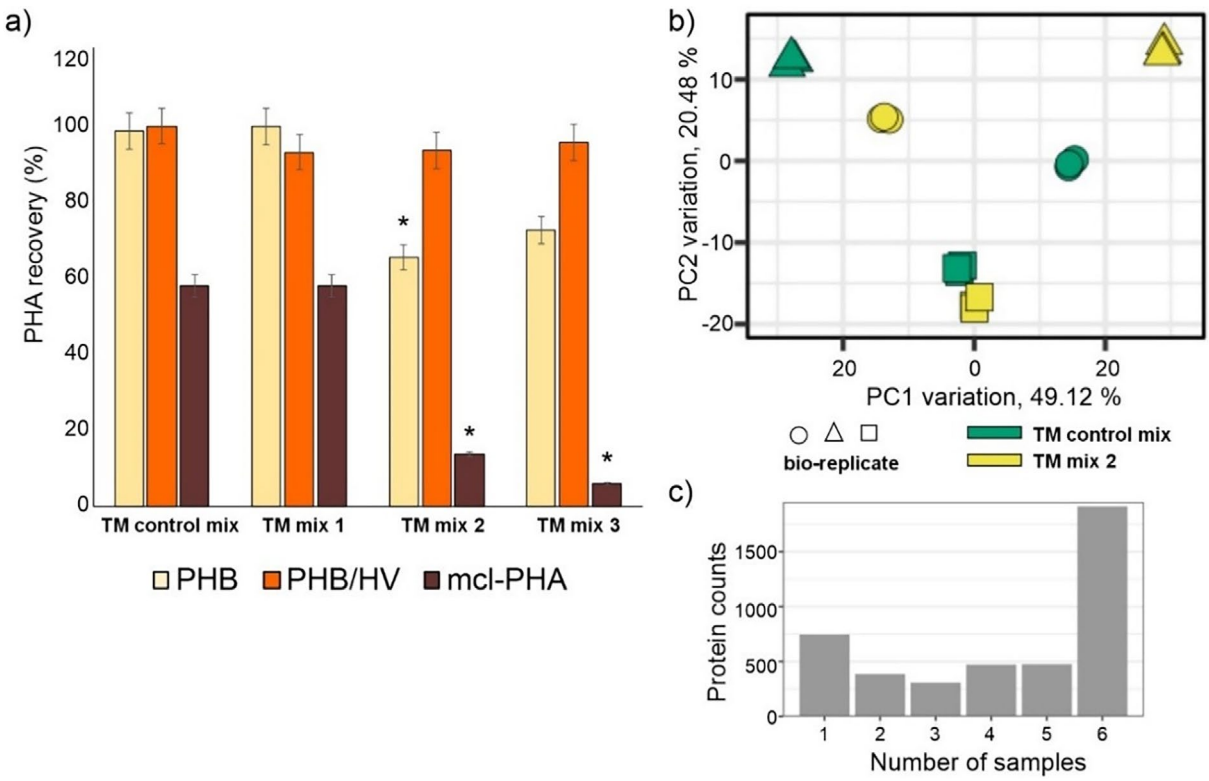

FIGURE 4 | PHA extraction using *T. molitor* protein mixes upon pregrowth on bran and bacterial biomass and proteome assessment. (a) TM control mix (100% bran); TM mix 1 (50% bran/50% PHB); TM mix 2 (100% PHB biomass); TM mix 3 (80% bran/20% mcl-PHA containing biomass), after 8 h incubation at 50°C in 50 mM Tris–HCl buffer pH 7.6 and 0.6 wt% load. Statistical analysis (ANOVA test and post hoc Fisher's LSD test; $*p \leq 0.05$ was considered statistically significant) was performed; (b) Principal component analysis (PCA) biplot of the first two principal components (PCs), which explain most of the variance in protein intensities. Technical replicates share the same colour and shape. (c) Protein identification overlap across biological replicates.

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For downstream analysis, technical replicates were mean-aggregated. Despite differential differences on SDS-PAGE, proteomic profiling did not detect qualitative differences in protein identities between protein mixes obtained from mealworms pre-grown on bran and TM mix 2.

The *T. molitor* genome encodes 14,259 protein-coding genes. Of these, 4299 were quantified in proteomic analysis, and only 514 of them were single-peptide identifications. After filtering for proteins present in at least two replicates of any condition, 3250 proteins were reliably quantified. This represents a significant depth of coverage and high identification rates. Most of these proteins were present in all six samples (Figure 4c). Only nine proteins were present in all replicates of one condition and absent from the other: eight were unique to the bran condition and one beta-glucuronidase-like protein (LOC138138159) was unique to the bacterial-biomass feeding condition (Table S1). The proteomes of the two feeding conditions were highly similar in both identification and quantification. The minimal Pearson correlation was 0.82 and PCA of protein intensities did not separate samples by feeding condition (Figure 4a,b), which indicates that the feeding strategy had only a minor impact on the global proteome. Consistent with this, differential abundance analysis identified no proteins with statistically significant differences between the two conditions. Given that feeding condition-specific changes were not observed, the focus shifted on absolute protein abundance, measured by iBAQ values (see Text S1). The most abundant proteins were structural proteins, mostly cuticle-related (Table S1). The *T. molitor* genome contains 2993 genes predicted to encode uncharacterized proteins, and only 493 (16.47%) of these were detected in our data. Coverage was higher for enzyme families, with approximately 38% of predicted peptidases and esterases and 51.23% of predicted oxidoreductases being quantified.

From the five α-amylases of the IPR006046 family, two were quantified, while seven of the 33 cathepsins were quantified. To assess whether these enzymes might contribute to PHA recovery, the α-amylases and cathepsins were compared against enzymes previously reported for PHA extraction (Table 2; bromelain, trypsin, savinase, pancreatic lipase, pancreatic α-amylase and endo-β-1,4-glucanase) via BLAST. The search revealed α-amylase (P56634) had >50% sequence identity to α-amylase used in PHA recovery and cathepsin (A0A0B5IPN6) with >40% identity to bromelain and were thus recombinantly expressed in *P. pastoris* and used in subsequent experiments.

3.6 | PHA Extraction With Recombinant α-Amylase and Cathepsin

Enzymes identified by proteomics and BLAST as the most prevalent in *T. molitor*, namely α-amylase (P56634) and cathepsin (A0A0B5IPN6), were used for PHB recovery from *Z. denitrificans* MW1 and mcl-PHA recovery from *P. putida* CA-3 using optimized recovery conditions. The SDS PAGE assessment showed a distinct band corresponding to α-amylase at ~63 kDa and cathepsin at 36 kDa that are in line with the expected molecular weight ranges (Figure S9). The slightly higher Mw of obtained α-amylase is due to retention of the leader peptide (12 kDa). Activity of purified α-amylase was confirmed on starch agar plates using the clear zone method (Figure S10). Recovery with recombinant α-amylase was 82% for PHB, 87% for PHB/HV and 98% for mcl-PHA, which is comparable or slightly higher in comparison to the commercial Novozyme α-amylase (Table 2). Recoveries obtained by recombinant cathepsin were significantly lower for PHB (41%) and 75% for PHB/HV, while the recovery of 94% for mcl-PHA was achieved.

Comparing PHB recovery with recombinant α-amylase at 82% to 99% obtained with crude protein TM control mix or 100% with crude TM mix 1 (Figure 4a) suggests that crude TM preparations were efficient due to synergistic activities of multiple enzymes, and their easy preparation has the potential to significantly reduce future PHB production costs. However, for mcl-PHA, both recombinant enzymes recovered 94%–98%, surpassing the TM control mix and TM mix 1 at ~60% (Figure 4a), offering alternative recombinant solutions for high yields of this PHA type.

3.7 | LCA Study Comparing the *T. molitor* Crude Protein Mix With the $CHCl_3$ and Commercial Enzymatic Methods

The environmental performance of the newly developed mild aqueous extraction method employing *T. molitor* crude proteins was evaluated against two reference approaches commonly used for the recovery of PHAs from bacterial biomass (Chen et al. 2025; Chida et al. 2025). The benchmarked methods included (a) solvent-based extraction with $CHCl_3$ and (b) a classical enzymatic route using commercial proteases in a Tris–HCl buffer. The assessment focused on process energy demand, greenhouse gas emissions expressed as CO_2 equivalents, solvent use, waste streams and overall environmental burden per 1 kg of purified PHA. All numerical values are based on harmonized process assumptions and mass-energy balances provided in Table S2. The functional unit was defined as the recovery of 1 kg of dry, purified PHA. System boundaries covered biomass handling, extraction, enzymatic or solvent-based digestion, separation, solvent and chemical recovery, final drying and waste management.

Three PHA recovery system scenarios were considered (Table 5). These begin with the acquisition or preparation of raw materials and materials necessary for the PHA recovery process. Impacts primarily include the electricity used, chemical components and waste generated, requiring disposal. Upstream burdens from enzymatic preparations (commercial or insect-derived), buffer production and electricity generation were included. Equipment construction was excluded due to a negligible contribution at the industrial scale. Energy demand differed markedly between the recovery approaches. Solvent-based extraction showed the highest requirement (65 $kWh \cdot kg^{-1}$ PHA), driven by biomass drying and solvent distillation. The classical enzymatic route required 24 $kWh \cdot kg^{-1}$ PHA, while the *T. molitor* method required only 12 $kWh \cdot kg^{-1}$ PHA. Assuming an emission factor of 0.40 kg $CO_2 \cdot kWh^{-1}$, the resulting energy-related emissions for the three methods were 26.0, 9.6 and 4.8 kg $CO_2 \cdot kg^{-1}$ PHA, respectively. The chloroform-based method required 12.5 L of $CHCl_3$ and 75 L of methanol per functional unit.

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Despite a recovery efficiency of 95%, solvent losses generated significant emissions due to the high carbon intensity of halogenated solvent production. The classical enzymatic method showed lower solvent consumption but still required large amounts of methanol. The *T. molitor* method did not require organic solvents, almost completely eliminating solvent emissions. When combining energy-related emissions, solvent loading, enzymatic contributions and waste treatment impacts, the total carbon footprints were 37.6 kg CO_2·kg^{-1} PHA for the $CHCl_3$ process, 15.7 kg CO_2·kg^{-1} PHA for the classical enzymatic method, and 5.0 kg CO_2·kg^{-1} PHA for the *T. molitor* method.

For all PHA recovery scenarios analyzed, a number of characteristics are presented and compared that may be important in decision-making processes because they are associated with specific environmental impacts (Table 6). The next stage of the environmental analysis involved a sensitivity analysis, which identified and modified key assumptions for the carbon footprint, analyzing their potential to change the final outcome. The chloroform method was strongly influenced by the solids content of the incoming biomass and solvent recovery efficiencies. Increasing fermentation broth solids from 10% to 20% decreased total impacts by nearly 24%, yet the method remained the least environmentally favourable. The classical enzymatic route was mainly affected by methanol recovery and the embodied impact of purified enzymes. For the *T. molitor* crude protein mix method, energy demand was the primary driver; even under conservative assumptions, the method remained significantly less burdensome than the alternatives.

Overall, the PHA recovery method using a mixture of crude proteins from *T. molitor* under mild aqueous conditions (0.6 wt% protein, pH ~7.6, 40°C) significantly reduces the carbon footprint compared to previous methods. The estimated carbon footprint for this method is approximately 5 kg CO_2·kg^{-1} PHA, assuming solvent-free operation and low levels of embedded enzymes. If purified embedded enzymes were used instead of raw flour, the total carbon footprint would be 5.75 kg CO_2·kg^{-1} PHA. If only the energy required for the process were 66.67% higher, the environmental impact would be 8.21 kg CO_2·kg^{-1} PHA, which is still more favourable than both scenarios. However, if the method requires a small amount of methanol (e.g., 10 kg MeOH), this translates to 12.01 kg CO_2·kg^{-1} PHA. Even with unfavourable assumptions in the sensitivity analysis, the result remains below the results of the other scenarios. Moreover, in the future, with the use of more precise environmental impact assessment methods such as Impact or ReCiPe—which take into account the toxicity of the ingredients and the residual loads resulting from the processes and equipment used—the benefits of the new *T. molitor* recovery method should receive additional, positive arguments and increase even further its applicability.

TABLE 5 | Summary of energy demand and carbon footprint for the three PHA recovery methods.

Method parameter	*T. molitor* crude protein	Classical enzymatic	$CHCl_3$
Total energy demand (kWh·kg^{-1} PHA)	12.0	24.0	65.0
Energy-related emissions (kg CO_2·kg^{-1} PHA)	4.8	9.6	26.0
Solvent/chemical embodied emissions (kg CO_2·kg^{-1} PHA)	~0.008	5.90	10.62
Waste treatment emissions (kg CO_2·kg^{-1} PHA)	0.2	0.2	1.0
Total carbon footprint (kg CO_2·kg^{-1} PHA)	5.0	15.7	37.6

4 | Discussion

4.1 | Enzymatic Disruption of PHA-Producing Bacteria and Polymer-Type-Dependent Recovery Behaviour

In this study, the effectiveness of different enzyme combinations for recovering PHAs from three distinct microbial biomasses was evaluated (Tables 1–3). The results demonstrate that selective enzymatic disruption of bacterial cells enables efficient recovery of intracellular PHAs directly from wet biomass, avoiding biomass drying and exposure to organic solvents. Recently, recovery of PHAs from wet biomass using acetone-water systems has been reported (Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025). In that study, water served as a non-toxic antisolvent replacing conventional organic precipitants; however, recovery yield, polymer purity and molecular weight distribution varied substantially

TABLE 6 | Key environmental characteristics of the assessed PHA recovery methods.

Method criterion	*T. molitor* crude protein	Classical enzymatic	$CHCl_3$
Use of hazardous solvents	Low (MeOH)	Moderate (MeOH)	High ($CHCl_3$, MeOH)
Need for biomass drying	No	No	Yes
Wastewater biodegradability	High	Moderate	Low
VOC emissions	None	Low–moderate	Significant
Scalability considerations	High (mild, aqueous)	Moderate	Limited (safety, solvent recovery)

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depending on copolymer composition, particularly HHx content, highlighting the sensitivity of polymer extraction to physicochemical properties (Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025).

Commercial enzymes such as alcalase, trypsin, pancreatin, bromelain and cellulase have previously been applied for enzymatic PHA recovery (Kapritchkoff et al. 2006; Marsudi 2006). While these approaches are generally effective for PHB extraction, additional purification steps using detergents or solvents are often required to reach the purity levels necessary for downstream applications such as biomedical materials or food packaging (Martino et al. 2014; Pagliano et al. 2021). Among the enzyme combinations evaluated here, the amylase–trypsin mixture yielded the highest recovery across all three biomass types (Table 2), highlighting the importance of synergistic enzymatic systems. Considering the relatively low bulk price of industrial-grade amylase compared with proteases such as trypsin or bromelain, incorporating amylase into enzymatic recovery processes could significantly improve the economic feasibility of enzymatic PHA extraction.

The superior performance of crude *Tenebrio molitor* enzyme extracts compared with individual commercial enzymes further indicates that complex multi-enzyme systems are required to effectively disrupt polymer-containing bacterial biomass. Gram-negative PHA producers such as *Z. denitrificans*, *C. necator* and *P. putida* possess multilayered cell envelopes consisting of outer membranes, peptidoglycan layers and intracellular protein matrices, which likely require coordinated hydrolytic activities for efficient degradation. The naturally balanced enzyme composition of *T. molitor* digestive extracts appears particularly well suited for this task, enabling efficient biomass solubilization under mild temperature and neutral pH conditions (Figure 2). The crude extract consistently outperformed individual enzymes in PHB recovery, supporting the concept that synergistic enzyme systems are more effective than single biocatalysts for processing structurally complex microbial biomass. This mirrors natural digestive systems, where heterogeneous substrates are degraded through coordinated enzyme consortia. From a biotechnological perspective, crude enzyme mixtures represent robust and potentially cost-effective alternatives to purified enzymes for industrial downstream processing compatible with large-scale fermentation systems.

T. molitor is usually considered as suitable and sustainable animal feed in the context of sustainable agriculture (Khanal et al. 2023; Zunzunegui et al. 2024). Proteomic analysis revealed a highly diverse enzyme repertoire in the *T. molitor* extract, with 3250 proteins reliably quantified. In addition to abundant structural proteins associated with the insect cuticle, carbohydrate-active enzymes and proteases represented a substantial fraction of the detected proteins. This observation is consistent with previous studies reporting high levels of cuticle proteins, hemolymph proteins and digestive enzymes in *T. molitor* larvae (Yi et al. 2016; Varunjikar et al. 2022). Recombinant expression confirmed that α-amylase and cathepsin contribute directly to polymer release, particularly in the case of mcl-PHA recovery. Digestive amylases from *T. molitor* have been known for a while to efficiently hydrolyse α-1,4-glycosidic bonds in starch-derived substrates under mild aqueous conditions (Buonocore et al. 1976), while digestive proteases such as cathepsins are abundant and display broad substrate tolerance and moderate thermostability (Elpidina et al. 2005; Beton et al. 2012). These enzymatic characteristics suggest that *T. molitor* represents a promising and sustainable source of biocatalysts for future development of biological biomass disruption strategies.

Overall recovery efficiency also differed between scl- and mcl-PHAs, highlighting the importance of polymer physicochemical properties in downstream processing. Scl-PHAs such as PHB and PHB/HV form intracellular granules that can take up to 80% of bacterial cells, that likely remain structurally cohesive during enzymatic biomass degradation. In contrast, mcl-PHAs are usually lower in numbers and can take up to 50% of producing cells, with a more dispersed intracellular distribution that may reduce accessibility during enzymatic lysis. Biomass composition also influences extraction efficiency. All microbial sources examined here exhibited high carbon content (Table 1), indicating dense organic biomass and polymer-rich intracellular environments. As Gram-negative bacteria, these organisms possess relatively thin peptidoglycan layers and outer membranes that are generally susceptible to enzymatic disruption. Differences in recovery between *Z. denitrificans* MW1 (70 wt % PHB) and *P. putida* CA-3 (49 wt % mcl-PHA) suggest that beside polymer characteristics such as monomer composition, type of bacterial biomass, recovery efficiency is also influenced by the overall PHA content (Rodrigues et al. 2022).

Biological lysis strategies have increasingly been investigated as alternatives to solvent-based PHA recovery. Conventional enzymatic digestion relies on purified enzymes such as proteases or lipases to degrade cellular components and release PHA granules, but often requires relatively high enzyme loadings and additional treatments such as surfactants or mechanical disruption to achieve efficient recovery (Pagliano et al. 2021; Zhang et al. 2024). Engineered self-lysis systems provide another strategy in which production strains express lytic enzymes that trigger controlled cell disruption at the end of fermentation, although these approaches require genetic modification and careful regulation to prevent premature cell lysis (Martínez et al. 2011). Similarly, predatory bacteria such as *Bdellovibrio bacteriovorus* can lyse Gram-negative hosts and release intracellular products, yet their use requires complex predator–prey cultivation and additional separation steps (Martínez et al. 2013). In contrast, the *T. molitor* enzyme system represents a distinct biological approach that exploits naturally evolved digestive enzyme mixtures capable of selectively degrading microbial biomass while preserving PHA granules. By utilizing crude insect-derived enzyme cocktails rather than purified enzymes, engineered strains or living predators, this strategy operates under mild aqueous conditions with low protein loadings and establishes a new class of biological downstream processing platform for solvent-reduced recovery of microbial biopolymers.

4.2 | Preservation of Polymer Molecular and Thermal Integrity

Maintaining polymer integrity is critical for high-value biomedical and packaging applications, where mechanical

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properties are closely linked to molecular weight and crystallinity. Importantly, enzymatic extraction using crude protein mix under mild aqueous conditions did not compromise polymer structure (Table 4, Figures S2–S5). Molecular weight distributions, polydispersity indices and NMR spectra confirmed that the chemical backbone of PHAs remained intact. In several cases, molecular weights were comparable to or slightly higher than those obtained through chloroform extraction, indicating minimal chain scission. The molecular weight distributions obtained in our study are consistent with those reported in the literature for PHAs (Nerkar et al. 2013; Reddy et al. 2022; Getino et al. 2025). Previous studies on PHB have reported Mn values of 80–100 kDa and Mw of 150–250 kDa, with PDI typically between 2.0 and 3.0, which closely resemble our results regardless of the extraction method used. Similarly, PHB/HV copolymers are usually reported to have Mn values between 50 and 100 kDa, Mw between 100 and 200 kDa and PDI between 1.5 and 2.5, which are also consistent with our findings. For mcl-PHAs, literature values are lower, with Mn typically 10–30 kDa, Mw 20–50 kDa and PDI 1.5–2.0, again comparable to the polymers recovered within this study. The polymers recovered here fall within these expected ranges, further indicating that enzymatic biomass disruption does not adversely affect polymer molecular architecture.

Thermal analysis confirmed that melting behaviour and crystallinity were also preserved. Observed variations were primarily attributable to intrinsic polymer composition rather than extraction-induced degradation. The presence of multiple melting features in PHB samples obtained with the TM control mix and α-amylase extraction is consistent with previous reports and likely reflects heterogeneous crystalline domains and heating-rate effects (Wellen et al. 2013). In contrast, the recovered mcl-PHA displayed a broad melting transition around ~78°C and very low crystallinity, reflecting its elastomeric character. In some cases, the TM control mix produced mcl-PHA with extremely low crystallinity and an almost absent melting peak, whereas α-amylase and chloroform extraction yielded weak but detectable melting transitions (Dartiailh et al. 2020). These findings demonstrate that selective enzymatic biomass hydrolysis enables physical recovery of native PHA granules without the polymer damage frequently associated with alkaline digestion or oxidative treatments.

4.3 | Environmental and Systems-Level Implications of Greener Downstream Processing Employing *T. molitor* Crude Protein Mix

Previous LCA studies consistently identify downstream processing as one of the most energy-intensive stages of PHA production. Solvent-based recovery is particularly penalized by high solvent-to-biomass ratios, energy demand for solvent recovery and the need for biomass drying prior to extraction, which substantially increases greenhouse gas emissions and operational costs (Saavedra del Oso et al. 2021). Reported carbon footprints for lysis-based PHA recovery processes range between 0.81–4.16 kg CO_2-eq kg^{-1} PHA, compared with 3.93–12.96 kg CO_2-eq kg^{-1} PHA for solvent-based routes, with chemicals and thermal energy representing the dominant contributors to environmental impact (Pagliano et al. 2021).

Within this context, the mild aqueous extraction strategy employing crude *T. molitor* proteins compares favourably with both solvent extraction and previously described chemical or enzymatic lysis approaches (Tables 5 and 6). The process eliminates halogenated solvents, avoiding solvent toxicity, VOC emissions and energy-intensive solvent recovery. In addition, it operates directly on wet biomass, bypassing drying, one of the major energy hotspots identified in previous LCA analyses (Khatun et al. 2025). Compared with classical enzymatic extraction, which often requires high enzyme loads and additional purification steps, the *T. molitor* system achieves comparable recovery efficiencies under mild aqueous conditions using low protein loadings. Relative to recently reported wet-biomass mechanical extraction using high-pressure homogenization that reached purities exceeding 95% and yields approaching 100% (Thiele, Gläser, et al. 2025; Thiele, Knorr, and Riedel 2025), the present approach provides a biologically driven alternative that minimizes mechanical energy input while preserving high polymer quality.

The environmental benefits observed here are therefore consistent with earlier process analyses. Solvent extraction carries a substantial energy burden due to solvent evaporation and recycling, whereas conventional cellular lysis often shifts impacts toward chemical consumption and wastewater treatment (Mannina et al. 2020; Pagliano et al. 2021). The *T. molitor* process reduces both burdens simultaneously by eliminating solvent recovery and decreasing thermal demand through wet-biomass processing while employing crude biological catalysts rather than large quantities of synthetic chemicals. This combination explains the markedly lower greenhouse gas emissions observed in this study and positions the process at the favourable end of the range reported for existing downstream strategies.

Sensitivity analysis further confirmed the robustness of this advantage. Even under conservative assumptions, the *T. molitor* route remained environmentally favourable, indicating that its benefits derive from overall process simplification rather than a single optimized parameter. In addition to lower carbon emissions, the absence of volatile solvents and the biodegradability of residual aqueous streams improve environmental compatibility and occupational safety. From a systems perspective, insect-derived enzymatic disruption therefore represents more than an alternative extraction method, it constitutes a promising biological downstream processing platform that could be integrated with fermentation-based PHA production. Future optimization may include strain-specific recombinant enzyme cocktails, coupling with low-energy separation technologies, or direct integration with wet-biomass fermentation streams to enable fully aqueous, modular PHA recovery processes.

5 | Conclusions

This study demonstrates that crude protein mixtures derived from *T. molitor* provide a robust, scalable and environmentally superior alternative to conventional solvent-based and enzyme-intensive routes for PHA recovery. Operating under mild aqueous conditions and using only 0.2 wt%–0.6 wt% crude protein, the process enabled near-quantitative recovery of PHB, high

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recovery of PHB/HV and competitive recovery of mcl-PHA directly from wet bacterial biomass, while fully preserving polymer molecular integrity and chemical structure. Importantly, the recovered PHAs exhibited molecular weights and polydispersities comparable to those obtained via $CHCl_3$ extraction, confirming that the new approach does not compromise material quality.

Proteomic analysis revealed that the exceptional performance of the crude *T. molitor* extract arises from the synergistic action of multiple digestive enzymes, rather than from a single dominant catalyst. Recombinant expression of α-amylase and cathepsin validated their key contributions, particularly for mcl-PHA recovery (94%–98%), yet also highlighted the advantage of multi-enzyme systems over isolated biocatalysts for efficient disruption of diverse microbial matrices. These findings underscore that PHA extraction efficiency is governed by a fine interplay between enzyme specificity, polymer physicochemical properties and cellular architecture, rather than by enzyme potency alone. The low wt% of crude proteins required is shifting away the burden of industrial enzyme manufacture toward low-impact insect cultivation.

From a sustainability perspective, LCA confirms that the *T. molitor*-based process delivers a step-change reduction in environmental burden, with a total carbon footprint of approximately 5 kg $CO_2 \cdot kg^{-1}$ PHA, representing a three-fold improvement over classical enzymatic routes and nearly an order-of-magnitude reduction relative to chloroform extraction. The elimination of halogenated solvents, avoidance of biomass drying, reduced energy demand and biodegradability of residual streams collectively position this method among the lowest-impact PHA recovery strategies reported to date.

Author Contributions

Evangelos Topakas: supervision, funding acquisition, writing – review and editing. **Brana Pantelic:** methodology, formal analysis, writing – review and editing. **Patrícia Concórdio-Reis:** writing – original draft, methodology, resources, writing – review and editing. **Filomena Freitas:** supervision, validation, writing – review and editing. **Marijana Ponjavic:** investigation, methodology, formal analysis, writing – review and editing. **Sanja Skaro Bogojevic:** investigation, methodology, visualization, writing – review and editing, formal analysis, writing – original draft. **Tatjana Ilic-Tomic:** methodology, investigation, writing – review and editing. **Dusan Milivojevic:** methodology, investigation, formal analysis, writing – review and editing. **Maciej Guzik:** resources, supervision, formal analysis, writing – review and editing. **Romanos Siaperas:** methodology, validation, formal analysis, writing – review and editing. **Jasmina Nikodinovic-Runic:** conceptualization, funding acquisition, writing – review and editing, supervision, formal analysis. **Mónica Carvalheira:** methodology, writing – review and editing. **Marcin Rychwalski:** methodology, formal analysis, writing – review and editing, writing – original draft.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this article have been included as part of the Supporting Information. Supporting Information (experimental procedures, MMC population identification, product characterization, ^{1}H NMR, ^{13}C NMR, DSC, SDS-PAGE analysis, protein quantification and life cycle assessment) is available. See DOI: 10.1039/x0xx00000x The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD072887. Data supporting this study are openly available from IMAGINE at https://imagine.imgge.bg.ac.rs/handle/123456789/3225.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** mbt270360-sup-0001-AppendixS1.docx. *Lampropedia*, with a typical tablet-shaped cluster, detected by FISH: LAMP444-targeted bacteria (in yellow) are *Lampropedia*; all other bacteria are in green. **Figure S2:** mbt270360-sup-0001-AppendixS1.docx. ^{1}H NMR spectra of PHB samples extracted with: (A) chloroform, (B) crude *T. molitor* control mix, (C) α-amylase. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.25 (dd, J = 6.43, 12.80 Hz, 1H); 2.53 (ddd, J = 6.60, 15.52, 21.43 Hz, 2H); 1.27 (d, J = 6.29 Hz, 3H) ppm. **Figure S3:** mbt270360-sup-0001-AppendixS1.docx. ^{13}C NMR spectrum of the PHB sample extracted with the crude *T. molitor* mix. ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.28; 67.76; 40.94; 19.90. **Figure S4:** mbt270360-sup-0001-AppendixS1.docx. ^{1}H-NMR spectrum of the PHB/HV sample extracted with the crude *T. molitor* mix. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.22 (m, 2H); 2.54 (m, 4H); 1.61 (m, 2H); 1.25 (dd, J = 6.70, 15.49 Hz, 3H); 0.90 (q, J = 7.06 Hz, 3H) ppm. **Figure S5:** mbt270360-sup-0001-AppendixS1.docx. ^{1}H NMR spectrum of the mcl-PHA sample extracted with the crude *T. molitor* mix. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.19 (dt, J = 5.90, 12.16, 3H); 2.53 (qd, J = 6.45, 15.43, 6H); 1.57 (s, 6H); 1.26 (s, 24H); 0.87 (t, J = 6.04 Hz, 9H) ppm. **Figure S6:** DSC thermograms of the extracted biopolymers: (a) PHB, (b) PHB/HV, (c) mcl-PHA. **Figure S7:** mbt270360-sup-0001-AppendixS1.docx. *T. molitor* grown under different cultivation conditions: (a) 100% bran (TM control mix); (b) 100% *Z. denitrificans* MW1; (c) 100% *C. necator* DSM 428 biomass (TM mix 2); (d) 100% *P. putida* CA-3 biomass. **Figure S8:** SDS-PAGE analysis used to achieve high-resolution separation of complex protein mixtures. 10 μL of samples at approximately 3.5 mg/mL concentration were loaded onto the gel: (1) 50% bran with 50% *C. necator* DSM 428 biomass (TM mix 1), (2) 100% *C. necator* DSM 428 biomass (TM mix 2), (3) 80% bran with 20% *P. putida* CA-3 biomass (TM mix 3), (4) 100% bran (TM control mix), (5) Blue Star protein marker. **Figure S9:** SDS-PAGE of recombinant α-amylase (a) and cathepsin (b) purified from *P. pastoris* X-33P. M: molecular weight marker (BlueStar Prestained Protein Marker, NIPPON Genetics EUROPE), S: Supernatant, FT: flow-through fraction, E1-E5: imidazole elution fractions collected during affinity chromatography. **Figure S10:** The enzymatic activity of recombinant α-amylase expressed in *P. pastoris* evaluated using a starch agar plate assay. Wells were loaded with different protein fractions obtained during purification: **K1**: flow-through fraction (non-binding proteins), **K2**: commercial α-amylase, 1–6: imidazole elution fractions collected during affinity chromatography. Following incubation, the plate was stained with iodine to visualize zones of starch hydrolysis. Clear halos around the wells indicate enzymatic degradation of starch, confirming α-amylase activity. **Table S1:** Proteins detected in all replicates of either the bacterial-biomass (BB) or the oat flakes (bran) (OF) condition and absent from the other. Numbers in columns BB_r1-3 and OF_r1-3 represent the average of relative protein intensities from LC–MS/MS analysis, expressed in arbitrary units (AU). **Table S2:** Harmonized mass-energy balances and environmental indicators per 1 kg purified PHA.

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