CHAPTER FIVE

Characterization of biodegradation of plastics in insect larvae

Wei-Min Wu* **and Craig S. Criddle**
Department of Civil & Environmental Engineering, Stanford University, Stanford, CA, United States
*Corresponding author: e-mail address: billwu@stanford.edu

Contents

Abstract

Biodegradation of plastics has been observed at rapid turnover rate by some insect larvae, especially those of *Coleoptera*, in particular *Tenebrionidae*. *Tenebrio molitor* larva is well studied and capable of biodegrading polystyrene (PS), polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC) in their digestive intestine in synergy with their gut microflora. This chapter includes the methods, protocols, and procedures used to characterize biodegradation of plastics in *T. molitor* larvae and their gut microbiomes with polystyrene as the model feedstock. The methods used can be expanded to enable investigation of other plastics and/or insects.

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1. Introduction

Synthetic polymers are widely used in daily life and by many industries with an annual global production of over 359 million tons in 2018 (Europe, 2019). The six most frequently used plastic polymers, are polyethylene (PE, 29.7%, including low-density PE (LDPE), and high-density PE(HDPE)), polypropylene (PP, 19.3%), polyvinyl chloride (PVC,10%), polyurethane (PUR, 7.9%), polyethylene terephthalate (PET, 7.7%) and polystyrene (PS, 6.4%). Disposal and accumulation of synthetic plastic wastes, with PE, PP, PVC, PUR, PET and PS as major components, have been an environmental concern for decades.

Researchers have investigated biodegradation of plastic wastes since the 1960s. Due to their highly stable C—C and C—H covalent bonds, high molecular weights, hydrophobic properties, and the absence of readily oxidizable and/or hydrolyzable groups, most plastic polymers are extremely resistant to biodegradation. In general, biodegradation of plastics involves at least three reactions: (a) depolymerization or cleavage of polymer chains; (b) formation of oxidized intermediates, and (c) mineralization of intermediates to CO_2 and H_2O and to Cl^- when PVC is a feedstock (Peng, Li, et al., 2020; Yang, Yang, Wu, Zhao, & Jiang, 2014).

Reports dating to the 1950s have implicated insect larvae in the penetration and deterioration of plastics (Cline, 1978; Gerhardt & Lindgren, 1954). Over the last 10 years, research has focused on insect larvae-mediated biodegradation of PS, LDPE, PP and PVC, within the darkling beetle families (Coleoptera: Tenebrionidae), *i.e., Tenebrio molitor*, *Tenebrio obscurus, Zophobas atratus*, *Tribolium castaneum* and *Plesiophthalmus davidis* (Peng, Li, et al., 2020; Peng et al., 2019; Wang, Xin, Shi, & Zhang, 2020; Woo, Song, & Cha, 2020; Yang et al., 2015a); larvae of pest moths (Lepidoptera: Pyralidae), *i.e.,* Indian mealmoths (*Plodia interpunctella*) (Yang et al., 2014), greater waxworms (*Galleria mellonella*) (Bombelli, Howe, & Bertocchini, 2017), and lesser waxworms (*Achroia grisella*) (Kundungal, Gangarapu, Sarangapani, Patchaiyappan, & Devipriya, 2019). Plastic biodegradation in these invertebrates occurs within hours during passage through their digestive tract, a rate that far exceeds those of microorganisms in laboratory cultures or in the environment where degradation occurs on the scale of weeks, months, or even years.

Darkling beetles are the common name for a large family of beetles (Tenebrionidae) that is cosmopolitan in distribution with more than 20,000 estimated members. To date, five species (*T. molitor*, *T. obscurus,*

Z. atratus, *Tribolium castaneum* and *Plesiophthalmus davidis*) are confirmed for degradation of PS and LDPE (Peng, Chen, et al., 2020; Peng et al., 2019; Wang et al., 2020; Woo et al., 2020; Yang et al., 2015a). In their native environment, larvae of the genus *Tenebrio* inhabit the decaying wood of forests (Calmont & Soldati, 2008) where they consume dried leaves containing lignocellulosic materials. These larvae are also able to biodegrade PS, LDPE, PVC and PP as well as vulcanized styrene-butadiene rubber (SBR) and tire crumb (Peng, Li, et al., 2020; Yang, Ding, et al., 2021a; Yang, Gao, et al., 2021b, 2015a). *Tenebrio molitor* has four life stages: egg, larva, pupa, and beetle (Roberson, 2005). *T. molitor* larvae typically measure about 2.5 cm or more, whereas adults are generally between 1.25 and 1.8 cm in length. After mating with the male, the female lays eggs. Over their lifespan, a female will, on average, lay about 500 tiny white eggs. The eggs hatch within 1–3 weeks. During the larval stage, the larva feed on wheat bran, vegetation and dead insects and molt, depending upon larval stage, or instar (they complete development with 9–20 instars, 180–270 days). After the final molt, the larva become pupa for 7 days or more (up to 20 days at lower temperatures). The pupa then become adult beetles for 30–90 days. The new beetle is whitish and turns brown over time.

2. Biodegradation of plastics in *Tenebrio molitor*

2.1 Incubation conditions for biodegradation of plastics

Factors impacting and/or controlling plastic degradation in *T. molitor* larvae are directly associated with the properties of polymers and larval physiological conditions (growth, development stage), metabolic activities and structure of the gut microbiome as described below.

2.1.1 Plastic feedstock

All major plastics (PE, PE, PP, PVC, PET, PUR, *etc.*) as well polylactic acid (PLA) have been tested for biodegradation by *T. molitor* larvae in labs at Stanford University and collaborators. The rate of polymer degradation is influenced by chemical and physical structures, molecular weights, and degree of crystallinity. Polymer materials have higher molecular weights and a higher degree of crystallinity are more resistant to biodegradation. The larvae prefer plastic materials as foam, thin films or tiny particles (size less than their mouth). For large and rigid plastic materials, pretreatment (size reduction, conversion of crystalline to amorphous state) is needed.

Microplastic (MP) materials have a size <0.1 mm for *T. molitor*. A mixture of MPs plus wheat bran can be made with 1.6–2.0% jelly as adhesive to prepare co-diet.

2.1.2 Effect of co-diet

Composition of the diet or feedstock impacts plastic consumption rate and metabolic activities. When larvae are fed plastic polymers as sole diet, they can maintain plastic degradation and survive for 3–5 weeks, but with decreasing their body weight. This is because digestion of plastics can only provide energy and carbon for maintenance but nutrients necessary for growth are not provided by a plastic-only diet. Cannibalism is frequently observed among larvae, providing them with additional energy source and nutrients. Co-diets that include wheat bran, fruits, or vegetables facilitate plastic degradation, larval growth, and completion of life stages. A co-diet ratio of wheat bran *versus* plastics at 8 to 12 (w/w) is recommended (Yang, Brandon, et al., 2018).

2.1.3 Temperature and humidity

Temperature impacts mealworm survival and plastic consumption. *T. molitor* larvae from most countries (China, Europe and the USA) show optimal viability between 25 and 28 °C but the *T. molitor* larvae in South Asia can tolerate temperature >32 °C. Humidity is usually controlled at 60–80%.

2.1.4 Rearing density

For a plastic degradation test, a proper density of 2–3 larvae/cm^2 is recommended. *T. molitor* larvae exhibit competitive feeding behaviors.

2.2 Gut microbes

Biodegradation of plastics in insects is believed to be contributed by synergistic reactions of the digestive system (enzymes, digestive fluid, *etc.*) and gut microbes. The essential role of gut microbes in plastic degradation has been evaluated based on the changes in molecular weights with antibiotic suppression of gut microbes as opposed to controls without antibiotic treatment. To date, the results showed that when the gut microbes in *T. molitor* larvae were inhibited by gentamicin or mixed antibiotics, PS and PVC depolymerization basically or completely stopped; but LDPE and PP depolymerization still continued to a lesser degree or with a change in the depolymerization pattern (Yang, Brandon, et al., 2018; Yang, Ding, et al., 2021a; Yang, Gao, et al., 2021b). PS and PVC degradation are

categorized as gut microbe-dependent, while LDPE and PP are less dependent upon gut microbes and can even be gut microbe-independent. To date, plastic (PS and LDPE)-degrading bacterial cultures have been isolated from *T. molitor* and other darkling beetles (Wang et al., 2020; Yang et al., 2015a; Yin, Xu, & Zhou, 2020).

Characterization of gut microbiomes is commonly applied to understand the community shift, dynamics, and predominance during plastics biodegradation. Studies indicate that gut microbial community structures within the gut of *T. molitor* larvae are diverse, and shift depending upon dietary changes (Brandon et al., 2018; Yang, Wu, et al., 2018). Feeding plastics such as LDPE and PS shifts the gut microbiome to a community that can biodegrade LDPE / PS (Brandon et al., 2018; Yang, Gao, et al., 2021b). Shifts in community structure are quantified using ecological metrics of richness - the number of species in the community- and by the evenness and dominance of species, as quantified by the Shannon and Simpson indices, metrics accounting for differences in the relative abundance of species: the fact that some species are common while others are rare. Principal coordinate analyses (PCoA) by diet (with and without plastic, for example) can be quantified using the Bray-Curtis dissimilarity index and used to determine how diet affects community structure at the levels of genera and species. Microorganism types most closely associated with plastic biodegradation can be identified at the level of genera and species.

Two sampling methods have been used to collect gut microbial samples, *i.e.*, rinsing of extracted gut tissue to collect gut microflora in phosphate buffered water (pH7.0) for DNA extraction (Brandon et al., 2018) and collecting intestinal tissue for DNA extraction (Yang, Wu, et al., 2018). The latter method can collect all of the microorganisms including those attached to the intestinal wall but also collect insect DNA that can interfere with sequence analysis and data processing. For current sequencing, the Illumina MiSeq platform is recommended for 16S rRNA gene amplicon, PacBio and NanoPore for full-length 16S rRNA genes, and HiSeq, NextSeq, and NovaSeq platforms for shot-gun metagenome sequencing. Detailed methods for gut microbiome analysis are provided in the references (Brandon et al., 2018; Peng et al., 2019; Yang, Gao, et al., 2021b).

2.3 Enrichment and isolation of plastic-degrading gut microbes

Isolation and characterization of plastic-degrading microbial species from insect larvae are needed to understand plastic-degradation and to identify

and characterize plastic-degrading enzymes. To date, plastic (*e.g.*, PS, LDPE)-degrading bacterial strains have been isolated from guts of darkling beetles (Yang et al., 2015b; Yin et al., 2020) and moth larvae (Yang et al., 2014). Reported bacterial cultures include LDPE-degrading *Bacillus* sp. YP1 and *Enterobacter asburiae* YT1 (Yang et al., 2014), LDPE-degrading co-culture of *Acinetobacter* sp. strain NyZ450 and *Bacillus* sp. strain NyZ451 (Yin et al., 2020), and PS-degrading *Exiguobacterium* sp. strain YT2 (Yang et al., 2015b) and *Acinetobacterium* sp. (Wang et al., 2020). Isolation methods are described in Section 4.

3. Analytical methods for plastic biodegradation

To characterize biodegradation of plastics by insects and their gut microorganisms, analytical methods are commonly used to determine mass loss, physical and chemical modification, changes in chemical structure, and biodegraded intermediates and products. Methods commonly used in published reports are summarized as below.

3.1 Gravimetric determination of weight loss

Weight loss is a widely used and essential indicator of biodegradation. To achieve accurate results, care is required in the selection of the proper mass of plastics material, clean-up of residual plastics, and recovery of residual polymer from frass *via.* solvent extraction.

3.2 Chemical and structure modification

3.2.1 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) and high temperature gel permeation chromatography (HT-GPC) are essential tools used to study depolymerization and biodegradation, and provide information on the relative molecular weight of polymer samples and the distribution of molecular weights. If comparable standards are used (PS is a common standard), the molecular weights determined can be within $\pm 5\%$ accuracy. Outputs include: the number average molecular weight (M_n), the weight average molecular weight (M_w), the size average molecular weight (M_z), and the viscosity average molecular weight (M_v). In general, M_n provides information about the lowest molecular weight fraction, M_w is the average closest to the center of the distribution curve, and M_z represents the highest molecular weight portion. M_v represents the viscosity of the polymer sample based on the relationship using an empirical Mark-Houwink equation. In a typical

distribution curve, the average values are related to each other as follows: $M_n < M_v < M_w < M_z$. The dispersity (also known as the polydispersity index or PDI) of a sample is defined as $PDI = M_n / M_w$ and gives an indication broadness of the polymer distribution. As results of biodegradation, significant changes in M_n, M_v, M_w and M_z will be observed; and the changes in MWD and PDI also occur. The method is not highly sensitive because the analysis is performed on the bulk polymer and cannot determine initial minor changes in biodegradation

3.2.2 Fourier transform infrared spectroscopy (FTIR)

FITR is commonly used to characterize chemical modifications of polymer structure and to monitor chemical changes on the polymer surface by detecting the formation of functional groups resulting from biodegradation or enzymatic attack. This method works well for the plastics with little or low additive content. To test biodegradation of plastics by insects, it is preferable to use the frass sample of the insect fed plastics as the sole diet. For tests with bacterial or fungal cultures, complete removal of biofilm or attached biomass on the surface of residual plastics is mandatory to avoid misleading results. Pretreatment with ultrasound plus 2% sodium dodecyl sulfate (SDS) is commonly used

3.2.3 Thermogravimetrical analysis (TGA)

TGA determines the thermal stability of a polymer. Biodegradation of plastics results in the decrease of the stability as an indicator. When frass of insects is tested using TGA, the samples containing co-diet such as wheat bran, starch, *etc.* could cause significant misinterpretation of results. A TG analyzer equipped with Fourier transform infrared spectroscopy (TG-FTIR) is commonly used to identify decomposition products from samples. Newly produced components from residual polymer sample are indicators of biodegradation

3.2.4 Proton nuclear magnetic resonance (^{1}H NMR)

^{1}H NMR is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the molecular structure. It has been used to study the chemical modifications of the polymers after biodegradation based on the formation of new peak(s) in the spectra of samples of biotreated polymers

3.2.5 Differential scanning calorimetric (DSC) analysis

DSC is useful for assessment of different thermal properties of materials and investigation of the thermal transition of synthetic polymers, such as glass transition (Tg). The shifts of Tg to lower temperatures indicates a decrease of stability of the polymer as a result of biodegradation. DSC can also be used for determination of M_v and crystallinity

3.3 Surface observation

3.3.1 Microscopic observations

This type of method is useful to observe the activities of gut microbes. Formation of biofilm or microbial attachment on plastic polymer surface can be observed using high magnification microscopes and fluorescence microscopes. Staining with the LIVE/DEAD BacLight Bacterial Viability Kit or other reagents can be used to observe or count live and dead cells. Biodegradation of plastic polymers by microorganisms has been evaluated using scanning electron microscopy (SEM) for years. SEM can be used for direct observation of microbial colonization on the plastic surface and formation of cracks and holes on polymer samples. However, colonization of polymer surfaces is not sufficient proof of the biodegradation given that microorganisms can grow on inert media as a support for biofilm formation and may survive by consuming biodegradable additives (such as plasticizers).

3.3.2 Water contact angle (WCA)

Determination of the WCA of the plastic polymer surface is commonly used to monitor the changes in hydrophobicity on polymer surfaces. Decrease in hydrophobicity can be an indicator of biodegradation activity and can be used to characterize the activities of gut microbes and gut digestive enzyme(s). Prior to the analysis, polymer samples must be cleansed to remove the biofilm and attached organics using SDS (2%, w/v) for at least 4 h prior to conducting a WCA measurement.

3.4 Isotopic labeling and other methods

3.4.1 Isotopic labeling

The biodegradation and mineralization of plastics can be assessed using labeled ^{14}C carbon in synthetic polymers. The production of $^{14}CO_2$ and $^{14}CH_4$ (if tested anaerobically) upon degradation is a direct indicator of mineralization. The release of soluble ^{14}C containing organics is also an indicator of biodegraded intermediates. ^{13}C labeled isotopic polymers are also used to examine biodegradation and mineralization by measuring $\delta^{13}C$ in the

CO_2 released and ^{13}C in biomass components such as fatty acids (Yang et al., 2015a). Biodegradation of ^{13}C labeled polymer will result in a significant increase in $\delta^{13}C$ in the released CO_2 and corresponding effects on biomass composition. The isotopic labeling is effective and powerful, especially employing ^{14}C but the application is limited due to difficulties and costs of preparing isotopic polymers and measures required for management and access to equipment.

3.4.2 CO_2 and methane production-based biodegradation methods

American Society for Testing and Materials (ASTM) and International Standards Organization (ISO) have developed methods for the evaluation of the biodegradation of plastics/polymers under aerobic or anaerobic conditions. According to ASTM, biodegradable means that the material can undergo decomposition into CO_2, CH_4, H_2O, inorganic compounds, or biomass. ISO definition requests only a chemical change of material (*e.g.*, oxidation). ASTM methods include tests for biodegradation under liquid, compost and solid conditions (see ASTM D5210–92(2007), D5338–11, D5511–2, D5526–12, D5988-12D6691–09). Although these methods were developed for examination of biodegradability of biodegradable polymers, they can also be used to evaluate enrichment-mediated or isolate-mediated biodegradation of plastic polymers and further degradation of plastic-containing frass.

3.4.3 Other methods

Other techniques have also been reported to evaluate biodegradation of plastics, including X-ray photoelectron spectroscopy (XPS), which was used to verify the changes in surface chemical components and functional groups (Yang et al., 2015b); solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR), which was used to identify newly appearing functional groups in solid samples (Yang et al., 2015a); attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), which identifies new functional groups in either liquid or sold samples during microbial biodegradation of plastics (Yin et al., 2020).

4. Protocols for the characterization of plastic degradation by *T. molitor* larvae

The following protocols focus on biodegradation of polystyrene (PS) in *T. molitor* larvae. They can also be employed for the degradation of other

plastics as noted in the text using other members of darkling beetle larvae *(e.g., T. obscurus, Z. atratus, etc.)*. These protocols include methods for the physiological characterization of the larvae responding to plastics, characterization of plastic degradation, sampling for gut microbiome, and isolation of plastic-degrading bacteria. To characterize biodegradation, analyses with GPC, FTIR, ^{1}H NMR and TGA can provide valuable results. SEM and WCA are used for gut microbial degradation studies.

4.1 Equipment, supplies and chemicals

4.1.1 Equipment and supplies

- Analytical balances.
- Centrifuge (10,000 rpm, 1.5 mL PP tubes).
- Environment incubator with temperature control (20–30 °C) and humidity displays.
- Freezer (−80 °C) to store DNA samples.
- Food grade polypropylene or polyethylene containers (300–500 mL in volume) suitable for the incubator.
- Glass vials with cap containing Teflon coated septum for DNA sampling.
- Glass vials with Teflon septum cover (5–10 mL) for polymer extraction.
- Norm-Ject 1 mL Polypropylene Luer Slip Tip Capsule Syringe for transfer THF extraction.
- PVDF (0.22 μm) sterile syringe filter (Thermo Fisher Scientific Inc., Dublin, Ireland).
- Refrigerator for frass samples and extracts.
- *Tenebrio molitor* larvae from online or local pet store with medium size (around 1.5 cm) or with an average weight of 60–70 mg each. The larvae should be fed on wheat bran without antibiotic supplements. The larvae should be healthy and contain no black dead larvae.

4.1.2 Chemicals

- Absolute ethanol (>99.5% purity, w/w).
- Plastic feedstocks in foam or MPs (<0.15 mm): polystyrene (PS), high density polyethylene (HDPE), low density polyethylene (LDPE), polypropylene (PP), polylactic acid (PLA), polyethylene terephthalate (PET), and polyvinyl chloride (PVC).
- Sodium dodecyl sulfate (SDS) used for the clean-up of plastic surface.
- Soy protein from nutrition store as nutrition-rich co-diet.
- Tetrahydrofuran (THF) used for extraction of PS and PVC.

- 1,2 Dichlorobenzene (DCB), >99.9% purity (w/w), used for the GPC analysis of LDPE and PP. DCB is considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200).
- Dichloromethane (DCM) >99.9% (w/w) for LDPE, PP and PLA extraction. DCM is considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200).
- Wheat bran from any pet store or farmer's market.
- Jelly powders or Jello (sugar free) from food store.
- Chemicals (analytical grade) for microbial media include: KH_2PO_4, K_2HPO_4, $MgSO_4$, NH_4NO_3, NaCl, $FeSO_4{\cdot}7H_2O$, $ZnSO_4$, $MnSO_4{\cdot}H_2O$; and agar, bacteriological tryptone, yeast extract.

4.2 Operational protocols

The following protocols are described for a test of biodegradation of PS foam or PS MPs in *T. molitor* larvae with and without wheat bran as co-diet. They can be modified for LDPE, HDPE, PP, PVC, PET, and PLA, and supplementing different mass ratios of co-diet. Other co-diet, *e.g.,* soy protein powders can also be considered.

4.2.1 Feedstock preparation

Plastic foam from manufacture can be used as feedstock directly. PS foams include expanded PS foam (EPS), which is used for building insulation and packing, and extracted PS foam (XPS), which is used as cups and food trays. Measure the density of the foam (g/cm^3) prior to use and contact manufacturer to understand the composition of additives in the foam. GPC analysis (see Section 3.2.1) is needed to determine the molecular weights and molecular weight distribution (MWD).

PS MPs can be used as feedstock. Jelly powders is used as adhesive to make PS-bran mixed diets. The procedures are:

1. The optimal mixture for the larval growth is at a ratio of bran/PS of 5:1–8:1 (Yang, Brandon, et al., 2018) but the ratio may be changed depending on tests.
2. Jelly powder (0.5 g) is dissolved into 30 mL boiled deionized water in a 200 mL glass beaker.
3. PS MPs (5 g) is added into the beaker.
4. After 10 min of stirring, the beaker is transferred to −4 °C refrigerator to mature the mixture for 1 h.
5. Finally, wheat bran (25 g) is added in the beaker and mixed completely with the solidified jell.

6. The mixture is then put in an aluminum pan and turned to air-drying for 2 days prior to feeding the larvae.
7. To prepare the control with wheat bran only, 30 g bran is mixed with 0.5 g Jelly.
8. Different mass ratios of wheat bran *versus* PS or other plastic MPs are prepared using the same procedures with a total 30 g weight of mixture plus 0.5 g jelly.

4.2.2 Set up of incubators with plastic foam as target feedstock

1. Add 300–350 larvae in each container at 25 °C, humidity of 70%.
2. Treatments include, *e.g.*, (i) unfed control; (ii) bran-fed control; (iii) PS only fed; (vi) PS plus bran-fed.
3. Duplicate each treatment.
4. Initial feedstock dosage: PS foam, 2 g; and wheat bran, 2 g.
5. Supplement wheat bran (2 g) once per week.
6. Supplement PS foam (2 g) when it is nearly consumed.
7. The incubation period for a short-term test is 5 weeks, and it can be up to 50 weeks for long-term test.
8. At the end of the test, collect all frass, weigh it, measure the dry weights, and determine content of residual PS (%, w/w) in frass to calculate PS mass balance.

4.2.3 Set up of incubators with microplastics (MPs) as feedstock

1. Add 300–350 larvae in each container at 25 °C, humidity of 70%.
2. Treatments include, *e.g.*, (i) unfed control; (ii) bran-fed control; (iii) PS MPs only fed; (vi) PS MPs plus bran-fed.
3. Duplicate each treatment.
4. Initial feed stock dosage: PS MPs-bran mixture, 30 g; control wheat bran, 30 g.
5. Supplement the mixed feedstock as it is consumed by observation.
6. The incubation period for a short-term test is 5 weeks, and it can be up to 50 weeks for a long-term test.
7. At the end of the test, collect all frass, weigh it, measure the dry weights, and determine content of residual PS (%, w/w) for biodegradation/digestion mass balance.

4.2.4 Larval counting and frass collection

1. Count number of live larvae, dead larvae, pupae and beetles and record their respective weights once per week. Discharge dead larvae.

2. Move the live larvae from each incubator, cleanse the larvae using compressed air flushing and then transfer them to a clean PP container. Keep them over night to allow them egest frass in it in order to collect frass.
3. Transfer the larvae back to the original incubator.
4. Collect frass into a pre-weighed PP centrifuge tube (1.5 mL), weigh the frass and keep the tubes with frass at −20 °C for GPC and other analysis (FTIR, TGA, ^{1}H NMR).

4.2.5 Sampling for gut microbiome analysis

1. Prior to the test, take 15–20 larvae, weigh them, and record the average weight. Then, transfer them into a glass vial (20 mL), fill the vial with absolute ethanol (purity >99.5%) to kill the larvae, and seal the vial with ethanol. The sample serves as background control.
2. For a short-term test (5 weeks), sampling with 15 larvae for each treatment is conducted at the end of the test using the same method as above.
3. For a long-term test, multiple sampling is conducted during the incubation period (weekly or monthly) and at the end of test.
4. All specimen in ethanol are preserved at −80 °C prior to DNA extraction.

4.2.6 Calculation of physiological parameters of the larvae

Survival rate (SR), cannibal rate (CR), pupation rate (PR) and the total mass of frass generated are determined periodically and must be calculated or measured at the end of test. The number of live larvae was counted to calculate SR as:

$$\mathrm{SR}\ (\%) = (\mathrm{N_c})/\mathrm{N} \times 100\%$$

where N is initial number of the larvae, and N_c is the number of live larvae (plus pupae and beetles if found) on day c. The numbers of pupae and disappeared larvae are counted to calculate PR and CR accordingly.

$$\mathrm{PR}(\%) = \mathrm{N_p}/\mathrm{N} \times 100\%$$
$$\text{and } \mathrm{CR}(\%) = (\mathrm{N} - \mathrm{N_c} - \mathrm{N_d})/\mathrm{N} \times 100\%$$

where N_p is accumulated number of pupae, N_d is the number of dead larvae.

4.2.7 Residual fractions in frass

Determination of residual polymer content in frass egested from the larvae is a basic procedure to characterize biodegradation rate, extent, and mass balance, as part of the extraction of residual polymer for the GPC analysis.

The frass sample contains (i) water soluble fraction (soluble salts, dissolved organic products, *etc.*); (ii) ethanol extractable fraction (biodegraded intermediates, residues of co-diet, *etc.*); and (iii) solvent (THF, 1,2 DCB or DCM) extractable fraction. To conduct solvent extraction, THF is used to extract residual polymer of PS and PVC;1,2 DCB is used for LDPE and PP; and dichloromethane (DCM) is used for PLA, LDPE and PP.

For PS extraction, the following steps are used.

1. Frass sample (1.0 g) from each diet is first mixed with deionized water (20 mL) in a conical flask after ultrasonic treatment for 3 min and extracted in a shaker with 100 rpm for 3 h.
2. The water solution is filtered with a 0.22 μm poly(ether sulfone) (PES) membrane filter or fiber glass filter and then stored at 4 °C for any further analytical use.
3. After filtration, the residue is vacuum dried and weighed to calculate the mass of water-soluble fraction.
4. Subsequently, the residue is further mixed with ethanol (20 mL), collected ethanol-extracted fraction as above steps 1–3, in a 50 mL glass vial. Ethanol extracted solution is stored in glass vials capped with Teflon coated septum cover for any further use. The solid residue is air-dried and weighed.
5. Finally, the residue is mixed with solvent (THF, 20 mL) to obtain the THF-extractable fraction in a 50 mL glass vial capped with Teflon coated septum cover. THF extracted solution is stored in glass vials capped with Teflon coated septum cover for GPC analysis and/or further use.
6. After evaporation of THF, the residue solid is air-or vacuum dried, and weighed.
7. Calculation of water soluble-, ethanol extracted- and DCM-extractable fraction of frass as below:

$$C_w\ (g/g) = (W_f - W_w)/W_f \times 100\%$$

where C_w = water soluble fraction (%, w/w), W_f = weight of frass sample (g), W_w = weight of dried residual solid after water extraction (g).

$$C_e\ (\%) = (W_w - W_e)/W_f \times 100\%$$

where C_e = ethanol extracted fraction (%, w/w), W_e = weight of dried residual solid after ethanol extraction (g).

$$C_{THF}\ (\%) = (W_e - W_d)/W_f \times 100\%$$

where C_{THF} = THF extractable fraction (%, w/w), W_d = weight of vacuum dried residual solid after THF extraction (g).

The residual PS content in frass of the larvae fed with PS plus different ratios of wheat bran (x %, w/w) is calculated as.

$$C_{PS} = C_{dx} - C_{db} \times (100 - x)/100$$

where, C_{PS} is PS content in frass (%, w/w), C_{dx} is the THF extractable fraction of frass of the larvae consumed PS and bran at a ratio of x %. The x is the ratio calculated from mass loss of PS feedstock and bran (%, w/w), or x is the percentage of PS in the MPs-bran diet (%, w/w) when PS MPs are used to prepare PS-bran mixed feedstock. C_{db} is the THF extracted fraction of frass from the larvae fed on wheat bran only.

4.2.8 Polymer (PS) removal

Specific polymer (PS) consumption rate (SPCR) is calculated on the basis of the mass of PS consumed by 100 larvae over the test period as:

$$SPCR = W_{ps} \times 100/(N_a \times t)$$

where SPCR is specific polymer (PS) consumption rate (mg/100 larvae-d); Wps is the mass of total PS consumed (mg); Na is averaged number of the larvae over test period; t is the total days of test period.

At the end of the test, PS removal efficiency is calculated as.

$$\text{PS removal } (\%, w/w) = (W_{PS} - W_f \times C_{PLA})/W_{PS} \times 100\%$$

where, W_{PS} is the total weight of PS consumed by larvae, W_f is the total weight of frass generated (g), C_{PS} is the PS content (%, w/w) in the frass.

4.2.9 GPC analysis

Gel permeation chromatography (GPC) is applied to analyze changes of molecular weight of the PS polymer (*e.g.*, employing Agilent 1260, Agilent Technologies Inc., USA).

1. Preparation of samples of frass are similar to the THF extraction test described previously (4.2.7). A sample of PS feedstock is dissolved directly in THF.
2. PS feedstock (0.2–1.0 g) and frass samples (0.5–1.0 g) from PS-fed larvae are commonly used.
3. THF extraction is conducted in a 50 mL glass vial capped with Teflon coated septum cover overnight.

4. After filtration with a 0.22 μm PVDF sterile syringe filter (Thermo Fisher Scientific Inc., Dublin, Ireland), the THF solution is transferred into a pre-weighed 50 mL glass vial, mixed on a magnetic stirrer with gentle heating (60 °C) or vacuum-dried overnight.
5. The vial with dried PS residue is weighed to measure the mass of residual PS.
6. The PS residue is re-dissolved with THF to achieve a concentration of 5 mg/mL.
7. Finally, the THF solution is transferred into a 1.0 mL GC vial and sealed for further GPC analysis.
8. The injection volume and eluent used depends on the model of GPC or HT-GPC used.

4.2.10 FTIR Analysis

Fourier transform infrared spectroscopy (FTIR) is used to detect changes of major functional groups of residual PS in frass (using, *e.g.,* Nicolet iS05 FTIR Spectrometer, Thermo Fisher Scientific, U.S.A.).

1. Prior to the analyses, the frass sample is lyophilized for at least 36 h to avoid deviation from water.
2. Then the sample is grinded with KBr to prepare a homogenous KBr pellet for scanning. The concentration of the sample in KBr should be in the range of 0.2% to 1%.
3. Depending on model of FTIR employed, for plastic samples, the peaks are identified in the range of 4000–500 cm^{-1} using, *e.g.,* OMNIC software (Thermo Fisher Scientific Inc., Pittsburgh, PA).

4.2.11 TGA analysis

Thermal gravimetric analysis (TGA) is conducted to characterize thermal changes from PS feedstock to the frass sample (using, *e.g.,* TA-Q500, TA Instruments, U.S.A.). The heating program included two different atmospheres in order to study pyrolysis of the sample under nitrogen and air ambience, respectively.

1. Frass sample (10 mg) and PS sample (10 mg) are first heated from ambient temperature to 800 °C at a heating rate of 20 °C/min under high-purity nitrogen ambience (99.999%, v/v).
2. Then cooled down to 500 °C.
3. Finally heated again to 800 °C under air ambience.
4. Differential scanning calorimetry was conducted to further study the crystallization temperature and melting process

4.2.12 ^{1}H NMR analysis

To characterize changes in the end groups of the egested polymer, liquid-state ^{1}H nuclear magnetic resonance (^{1}H NMR) analysis is conducted at ambient temperature.

1. The residual PS was extracted from frass with THF as described in Section 4.2.7, and air- or vacuum dried.
2. The dried residue from frass sample (50 mg) or PS feedstock is placed in a 10-mL glass vials and extracted for 2 h with 2 mL chloroform-D (purity 99.8%, Cambridge Isotope Laboratories, Inc., Tewksbury, MA).
3. Extracts are filtered through 0.22 μm PVDF filters and transferred to a clean 10-mL glass vial. Approximately 1 mL of extract is transferred into 5 mm NMR sample tube (Wilmad, LabGlass, Vineland, NJ, USA).
4. ^{1}H NMR spectra were measured on a 500-MHz NMR spectrometer (32 scans, delay time (d1) = 0.0 s). ^{1}H spectra are reported in parts per million (ppm) and referenced to a peak for residual deuterated chloroform (1H-7.26 ppm).

4.2.13 Water contact angle (WCA) analysis

1. Prior to the test, the plastic samples (pieces or films) are separated from microbial culture and rinsed with DI water.
2. The solid samples are mixed with 2% (w/v) sodium dodecyl sulfate (SDS) solution for 4 h and then rinsed with deionized water.
3. Check the sample carefully under a microscope and no visible biomass/biofilm should be observed. Otherwise, further clean-up is needed.
4. Measurement can be conducted using a contact angle measuring device (OCA40, DataPhysics, Filderstadt, Germany) or others.

4.2.14 Gut Microbial enrichment and bacterial isolation

1. Liquid carbon free basal medium (LCFBM) is used to enrich and characterize plastic-degrading gut bacteria. It contains (per 1000 mL deionized water) 0.7 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.7 g of $MgSO_4{\cdot}7H_2O$, 1.0 g of NH_4NO_3, 0.005 g of NaCl, 0.002 g of $FeSO_4{\cdot}7H_2O$, 0.002 g of $ZnSO_4{\cdot}7H_2O$, and 0.001 g of $MnSO_4{\cdot}H_2O$ according to the ASTM standard for determining the resistance of plastics to bacteria (G22–76, 1996).
2. After preparation, LCFBM (100 mL) is distributed in Erlenmeyer flask (250 mL), autoclaved at 121 °C for 40 min, then cooled prior to use.
3. The Luria-Bertaini (LB) medium contains (per 1000 mL deionized water) 10 g of bacteriological tryptone, 5 g of yeast extract, 10 g of NaCl and 15 g of agar. It is used for the isolation of single colonies.

4. LB is prepared in a 1000 flask with 500 mL Erlenmeyer flask according to the recipe, then add the desired amount of agar (normally about 1% w/v).
5. Autoclave LB for 25 min.
6. Cool the LB medium-agar mix to 55 °C.
7. Pour the plates. Use about 30 mL for each plate in a 100 mm diameter plate.
8. Allow the plates to set. Dry the plates in the laminar flow hood with the lid slightly off for 30 min (or in a 37 °C incubator for 2–3 h) prior to use.
9. If the plates are not used immediately seal them with Parafilm, or in the bag that the plates came in. Store the plates at 4 °C.
10. The whole guts of 10–15 larvae are extracted and pooled into a 10 mL centrifuge tube containing 5 mL saline solution.
11. After vibrating by a vortex for 5 min, the gut tissue is carefully removed, and the remaining suspension is moved into a 250 mL Erlenmeyer flask containing 100 mL LCFBM with 1 g PS micro powders (0.1–0.2 mm) or PS film pieces (1 cm × 1 cm) as the sole carbon source.
12. The mixture is incubated at 25 °C with shaking at 120–180 rpm for 15–60 days.
13. When turbidity occurs, the culture is serially diluted and spread on the LB agar plate to form single colonies at 25 °C.
14. Usually, emerged single colonies are observed within 24–72 h and then picked.
15. The picked colony is transferred in LCFBM with PS pieces or PS MPs for incubation.
16. Check morphotype under a microscope as cells grow (increase in turbidity).
17. Repeat steps 12–16 till single culture or same morphotype cells are observed.
18. Pure cultures can be obtained by repeatedly plate streaking. The abstained pure bacterial cultures are then further verified for PS degradation capacity in PS-containing LCFBM.

5. Results and analysis

T. molitor larvae actively chew, ingest and digest PS foam as well other major plastics (LDPE, PVC, PLA, PET, and PP) in either foam forms or MPs mixed with co-diet bran. The ingestion of plastics by the larvae impacts their physiology. Here we use the results of PS biodegradation as examples.

5.1 Larval activities and degradation rates

5.1.1 Survival rates and PS consumption of mealworms

T. molitor larvae chew and ingest PS foam regardless whether PS is supplied as sole feedstock or co-diets with wheat bran or soy protein or other nutrition-rich diet. At 25 °C, the larvae consumed PS (Fig. 1A) with much higher survival rates than unfed larvae in short term (Fig. 1B). One test showed that over a 32-day period, the SRs of the larvae are: unfed, 54.2 ± 2.5%; PS only, 86.7 ± 3.3%; PS plus bran, 90.0 ± 0.8%. During the test period, the larvae fed with PS (1.8 g) consumed 0.83 ± 0.04 g PS with average specific consumption rate of 11.8 mg ± 0.1 mg PS/100 larva per day. The unfed larvae lost 2.6 ± 0.2% of their average weight; the larvae fed with PS only maintained a stable weight; and bran-fed larvae experienced 32.0 ± 1.5% weight increase. The results indicated that ingestion of PS supports the life activities of the larvae but does not support their growth due to lack of necessary nutrition.

Another test with the co-diet with bran soy protein enhanced PS consumption significantly (Fig. 1C). Over a 32-day period, the larvae fed with PS only consumed PS at SPCR of 22.2 ± 1.8 mg/100 larvae per day but SCCR of the larvae fed co-diet bran and soy protein were 44.1 ± 4.8 and 49.1 ± 4.1 mg/100 larvae per day, respectively. The respective PS mass removal was 39.1 ± 1 3.6%, 67.6 ± 4.3% and 76.8 ± 2.8%.

A long-term test at 25 °C with 350 larvae for each treatment indicates that the diet conditions impact the survival rate (SR) pupariation rate (PR), and cannibal rate (CR). For the unfed group, the parameters were: SR, 4.0%; PR, 4.0%; CR, 96%. The larvae fed PS only had: SR, 9.7%; PR, 9.7%; CR,90%. The larvae fed PS plus bran showed: SR, 81%; PR, 75%; CR, 18%. The total mass of PS consumed was 1.13 g by the larvae fed on PS only *versus* 5.20 g by the larvae fed PS plus bran.

5.2 Characterization of biodegradation

5.2.1 THF extractable fraction in frass

THF extractable fraction from frass is used as indicator to estimate the content of residual PS in frass. Because PS (or other plastics) does not dissolve in water and ethanol, the previous two steps of extraction with DI water and ethanol can remove most organic contaminants, additives, and biodegraded intermediates from the frass. The THF extractable fraction is composed of mainly PS polymer with white color (Fig. 1D).

THF extractable fraction was measured on day 4, 8, 12, 16, 20,24, 28 and 32 during a 32-day test illustrated in Fig. 1B and D. The PS residue in frass (w/w, %) of the larvae fed PS only decreased from 66.2 ± 2.3% on day 4 to 35.2 ± 1.2% by day 24, stabilizing thereafter. The results indicate a progressively increased PS digestion capacity.

5.2.2 GPC analysis

GPC analysis provides information on three key indicators of depolymerization and degradation of plastic materials: $M_{n,}$ M_w and MWD. Here we use the results of the test in Fig. 1C as example. The samples were obtained for PS residues extracted from frass collected on day 4, 8, 12, 16, 20, 24, 28, and 32. GPC analysis of the extracted PS residues (Fig. 1 D) reveals a progressive shift of MWD from higher to lower molecular weights over time compared to the PS feedstock (control), showing a typical broad depolymerization pattern (Fig. 1F), with about 16 days required to reach relatively stable lower levels of M_w and M_n (Fig. 1H). M_n and M_w values for PS residues in the frass after 32 days are significantly lower than M_n and M_w for PS in the feed. M_n decreased from 95,800 ± 1300 to 77,000 ± 4000 ($P = 0.0214$); M_w decreased from 239,000 ± 2400 to 212,000 ± 4800 ($P = 0.0206$). The results (Fig. 1E and F) suggest that the PS degradation activity increased gradually but stabilized after a 16- to 24-day adaptation period.

Depolymerization is the essential step of biodegradation of plastic polymers. Two different depolymerization patterns have been observed during biodegradation of plastics by insect larvae and microbial cultures., broad depolymerization or the decrease in both M_n and M_w of the plastic polymer (Fig. 1F); and limited extent depolymerization or increase in M_n and decrease in M_w or increase in both M_w and M_n (Peng, Chen, et al., 2020; Peng, Li, et al., 2020; Yang, Ding, et al., 2021a). However, decrease in M_z has been observed in both patterns.

5.2.3 FTIR analysis of frass

Here we present typical FTIR spectra to determine how PS polymers are modified using the frass samples of the larvae fed with PS only, bran plus PS, and bran only. FTIR is analyzed in comparison with FTIR spectra of PS feedstock (Fig. 2A). The larvae were incubated under four diet conditions at 25 °C for 32 days. The intensities of the peaks at 625–970 cm^{-1} (ring-bending vibration) are strong in PS feedstock but much weaker in frass samples. Characteristic peaks known to represent the PS benzene ring (C═C stretch, 1550–1610 and 1800–2000 cm^{-1}) almost disappear in frass

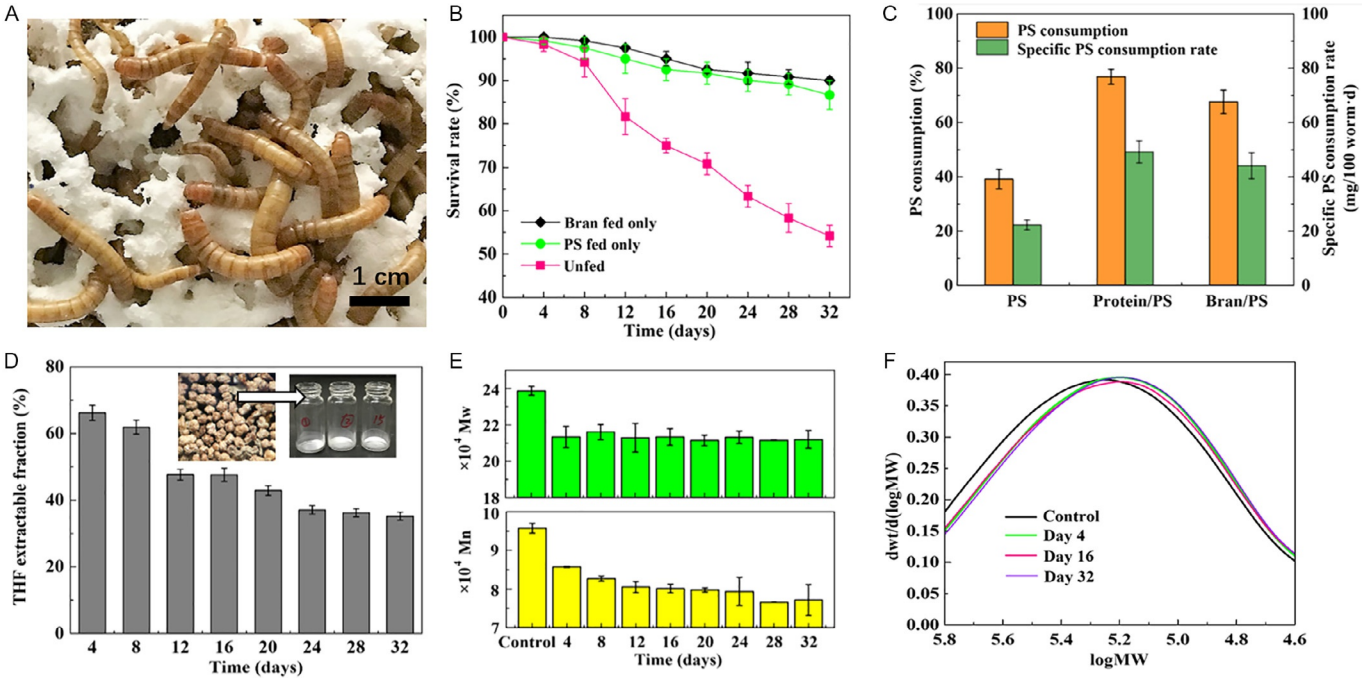

Fig. 1 (A) The larvae of *Tenebrio molitor* chew and ingest PS foam. (B) The survival rates of the larvae fed with different diets, *i.e.*, wheat bran plus PS, PS only and unfed. (C) PS consumption (%) and specific PS consumption rates with different co-diets over a 32-day period. (D) The decrease in HF extractable fraction (%) of frass indicating the enhanced digestion extent in larval guts. Insert photos: left is frass, right is the residual PS extracted from the frass with THF. (E) GPC analysis shows decrease in Mn and Mw of residual PS polymer extracted from the frass during the incubation period. (F) Shift in molecular weight distribution of residual PS polymer *versus* the original PS feedstock. *Data of panels (B–F) adapted from Yang, S.S., Brandon, A.M., Andrew Flanagan, J.C., Yang, J., Ning, D., Cai, S.Y. et al. (2018). Biodegradation of polystyrene wastes in yellow mealworms (larvae of* Tenebrio molitor *Linnaeus): Factors affecting biodegradation rates and the ability of polystyrene-fed larvae to complete their life cycle.* Chemosphere 191, *979–989.*

samples, providing evidence of ring cleavage. Further evidence of biodegradation is the observed decrease in intensities of peaks characteristic for PS and the appearance of carbonyl groups (C═O stretch, 1700 cm^{-1}). The broadening of peaks at 2500–3500 cm^{-1} in all FTIR spectra of frass samples is associated with the hydrogen bond of hydroxyl groups and/or carboxylic acid groups, suggesting a shift from hydrophobic to more hydrophilic surface properties.

5.2.4 ^{1}H NMR analysis

The same samples used for above FTIR analysis were extracted with chloroform-D for ^{1}H NMR analysis. The comparison of spectra for PS to the spectra of frass extracts reveals new peaks in the frass from the larvae fed PS only and PS plus bran (Fig. 2B). These peaks are detected in regions of chemical shift associated with -CH═CH-, -CH_2-, carbonyl (H_2C═O), and hydroxyl (-OH) groups. Their presence in PS residues of frass, but not control PS, is evidence of transformations within the larval gut, indicating new oxidized functional groups.

5.2.5 TGA analysis

TGA is used to detect the thermal modification of PS ingested by *T. molitor* larvae at the end of a test (Fig. 1C). PS foam had a 98.29% weight loss occurring at 360 °C ~ 480 °C, with the maximum decomposition rate temperature of about 450 °C (Fig. 2C). There are three decomposition stages of the frass fed PS: in the first stage, 6.62% weight loss occurred at 175 °C ~ 275 °C; in the second stage, 12.89% weight loss occurred at 275–360 °C; in the third stage, 53.12% weight loss occurred at 360–480 °C. The bran fed larvae frass has three decomposition stages: in the first stage, 14.46% weight loss occurred at 175–275 °C; in the second stage, 22.96% weight loss occurred at 275–360 °C; 18.11% weight loss occurred at 360–480 °C. Under the same heating program, PS fed frass has more decomposition stages than PS foam, indicating that the frass of *T. molitor* larvae contains new components other than PS foams. In the third stage, the weight loss rate of the frass is significantly lower than that of PS foams, indicating that the PS components in the frass are significantly reduced. Decompositions below 100 °C are classified as volatile organic compounds (intestinal secretions, carboxylic acid compounds from PS, *etc.*), while decomposition from 100 °C to 360 °C may be attributed to other organic waste and biodegradable residues. In the stage of 360–480 °C, the mass loss rate of PS is 98.29%, and the loss rate of the PS fed frass is 53.12%. The three-stage decomposition of PS fed larvae frass is similar to those of bran-fed larvae frass,

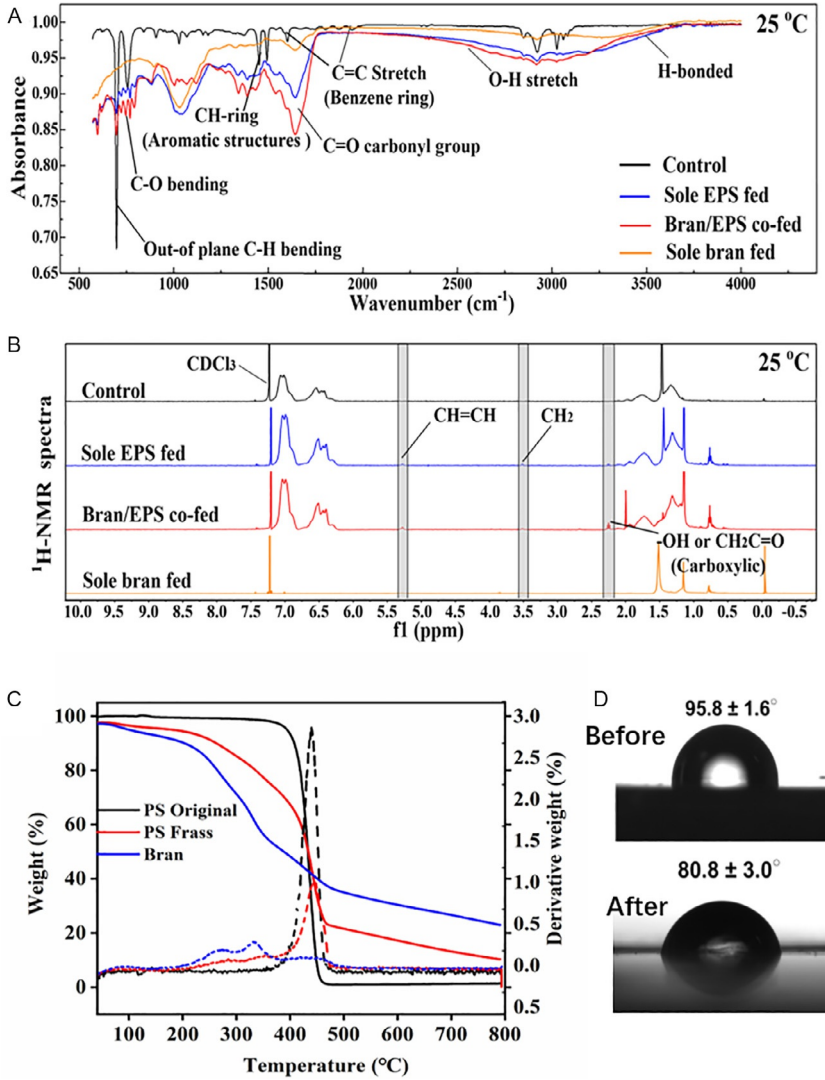

Fig. 2 (A) Spectra of FTIR and ^{1}H NMR of control (PS feedstock) *versus* frass samples of the larvae under different diet conditions, *i.e.,* fed with PS only, fed with PS plus bran and fed with bran only. The samples were collected after 32 days. (C) TGA analysis of PS feedstock *versus* the frass of the larva fed with PS only and bran only. (D) The change in WAC of PS pieces after incubation with strain YT1 for 60 days with 7.4% mass reduction. *Panels A–B adapted from Yang, S.S., Brandon, A.M., Andrew Flanagan, J.C., Yang, J., Ning, D., Cai, S.Y. et al. (2018). Biodegradation of polystyrene wastes in yellow mealworms (larvae of* Tenebrio molitor *Linnaeus): Factors affecting biodegradation rates and the ability of polystyrene-fed larvae to complete their life cycle.* Chemosphere, 191, *979–989. Panel C adapted from Yang, L., Gao, J., Liu, Y., Zhuang, G., Peng, X., Wu, W.-M., et al. (2021). Biodegradation of expanded polystyrene and low-density polyethylene foams in larvae of* Tenebrio molitor *Linnaeus (Coleoptera: Tenebrionidae): Broad versus limited extent depolymerization and microbe-dependence versus independence.* Chemosphere, 262, *127818. Panel D adapted from Yang, Y., Yang, J., Wu, W.M., Zhao, J., Song, Y.L., Gao, L.C. et al. (2015b). Biodegradation and mineralization of polystyrene by plastic-eating mealworms: Part 2. Role of gut microorganisms.* Environmental Science & Technology, 49, *12087–12093.*

indicating that PS fed larvae produced frass which has changed their composition to degraded products. The results indicate that PS is modified and oxidized after passage through *T. molitor* larvae. Similar TGA results were observed during biodegradation of LDPE and PVC in *T. molitor* larvae (Peng, Chen, et al., 2020; Peng, Li, et al., 2020; Yang, Gao, et al., 2021b).

5.3 Plastic-degraders

The presence of gut plastic-degraders provides evidence of plastic degradation in the larval guts. Plastic degrading bacteria were isolated using the methods described above by several researchers, *e.g.,* PS-degrading *Exiguobacterium* sp. strain YT2 (CGMCCC 10521) which grow on PS pieces as sole carbon source (Yang et al., 2015b). A total 13 single bacterial cultures were isolated by picking up colonies formed on LB agar plates and characterization of PS degrading capacity showed that *Exiguobacterium* sp. strain YT2 removed $7.4 \pm 0.4\%$ of mass of PS pieces over a 60 day period in LCFBM. Biodegradation was confirmed by decrease M_n from 40,400 to 37,500 Da and M_w from 124,200 to 110,100 Da using GPC analysis, formation of oxidized functional groups -C-O- by XPS, and decrease in WCA from $95 \pm 1.6\%$ to $80.8 \pm 3.0\%$. (Fig. 2.D).

Using the same methods with LDPE powders as sole carbon source, PE-degrading bacterial strains were also isolated from *Plodia interpunctella*, *e.g., Enterobacter absburiae* strain YT1 (GenBank KJ466896) and *Bacillus* sp. YP1 (KJ466897), growing on LDPE as sole carbon source (Yang et al., 2014); and *Acinetobacter* sp. strain NyZ450 (GenBank MT459299) and *Bacillus* sp. strain NyZ451 (MT459300), which can grow as co-culture on LDPE as sole carbon source (Yin et al., 2020).

6. Summary

Characterization of rapid biodegradation of plastics in insect larvae is described with a focus on *Tenebrio molitor* and its gut microbiome. The essential methods and protocols, and procedures are described to assess impacts on insect physiology, plastic mass balance, analysis of changes in polymer structure modification and oxidation, and isolation plastic-degrading bacterial species within the insect gut. The results of plastic degradation in *T. molitor* and isolation of plastic-degrading bacterial cultures are analyzed using examples. We envision that the methods used can be expanded to enable investigation of other synthetic polymer types and a broad assay of insects.

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