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RESEARCH ARTICLE

Efficient biodegradation of polyethylene (HDPE) waste by the plastic-eating lesser waxworm (*Achroia grisella*)

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Abstract

Polyethylene (PE) is one of the major persistent plastic that is not biodegradable at considerable rates in most environments, and is the major source of unceasing environmental pollution. Recently, biodegradation of plastic wastes through waxworms and mealworms were reported. The present study focuses on the high-density polyethylene (HDPE) degradation capabilities of the larvae of *Achroia grisella* (lesser waxworm) and its ability to complete its life cycle when fed with HDPE. Effects of added nutrition on PE degradation were assessed, providing wax comb as co-feed (PE-WC). The egested frass of the waxworm fed on waxcomb (WC), PE, and PE-WC were studied by analyzing the changes in physiochemical properties through FTIR and ^{1}H NMR techniques in addition to weight loss percentage of PE and survival rates of the tested lesser waxworms. The post-degradation studies of WC and PE showed 90.5 ± 1.2% and 43.3 ± 1.6% weight loss, respectively, by a group of 100 lesser waxworms. Over an 8-day period, PE consumption increased with an ingestion of 1.83 mg of PE per day per larvae. Supplementing the PE feed of lesser waxworms with WC facilitated enhanced PE degradation showing 69.6 ± 3.2% weight loss. Twenty-eight day survival rates for lesser waxworms fed on WC, PE, and PE-WC were 91.3 ± 1.01%, 74.6 ± 2.9%, and 86 ± 1.4%, respectively. The FTIR and ^{1}H NMR analysis of egested frass indicated formation of new functional organic groups, supporting biodegradation of PE in lesser waxworms. The frass of the lesser waxworm fed on PE samples shows the presence of new carbonyl and alcoholic groups with increase in unsaturated hydrocarbon indicating formation of biodegraded intermediates. Lesser waxworms fed with WC, PE, and PE-WC completed all life cycle stages (larvae, pupae, moth, and egg) developing into a second generation. The second generation of PE-WC fed larvae of *A. grisella* efficiently degrades PE at par with first generation counterparts.

Keywords Plastic wastes · Polyethylene · Biodegradation · Waxworm · *Achroia grisella*

Introduction

Plastics are synthetic polymers derived from fossil fuels enduring increased environmental scrutiny. Extensive production and use of plastic dates back to 1950 and presently the global production of plastic has outgrown to 322 million Mt per year (Worm et al. 2017). The properties like great durability and resistance to degradation make plastic highly versatile in innumerable applications. It is used mainly in the form of wrapping materials, packaging films, carry bags, fluid containers, toys, clothing, industrial products, building materials, and engineering and household applications. The tremendous consumption of plastic has resulted in the production of huge amounts of plastic waste, which has triggered a global environmental concern (Barnes et al. 2009; Law et al. 2010; Rochman et al. 2013; Wagner et al. 2014; Cózar et al. 2014; Jambeck et al. 2015). Plastic wastes ultimately make their way to water bodies and landfill sites and remain in the environment for several decades polluting the environment. As of 2015, out of 6300 million Mt of plastic waste generated, 79% was accumulated in the natural environment (oceans and river system) or in landfills (Geyer et al. 2017).

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Polyethylene (PE) is a common petroleum-based plastic, is expressed as "$[CH_2 - CH_2]_n$," and had been considered as non-biodegradable in nature due to its extreme durability and resistance to degradation. The inherent resistance of polyethylene to biodegradation stems from its three-dimensional structure, high molecular weight, hydrophobic nature, and lack of functional groups recognizable by microbial enzymatic systems (Harshvardhan and Jha 2013). Conventional methods used for polyethylene waste degradation including landfill, incineration, chemical treatment, and thermal degradation are lethal to the environment causing hazardous effects on living organisms (Yang et al. 2011). Direct incineration of polyethylene waste produces vapors which includes many toxic carcinogenic compounds like ketones, acrolein, and greenhouse gases like methane which causes serious health and environmental hazards (Briassoulis et al. 2004; Briassoulis 2006). PE wastes buried in soil may affect the drainage patterns, disturb soil fauna, and decrease the soil quality leading to declined agricultural yield (Ali et al. 2016). To combat this mounting problem, there has been a steady increase in research on PE biodegradation by fungi and bacteria. Many studies have been conducted on investigation of biodegradation of PE in natural environmental conditions including seawater, soil, compost, and sludge (Albertsson et al. 1987; Karlsson et al. 1988; Lee et al. 1991; Hadad et al. 2005; Kumar et al. 2007; Sudhakar et al. 2008; Nanda et al. 2010). The microbial species associated with the biodegradation of polyethylene include *Rhodococcus ruber* (Sivan et al. 2006), *Bacillus sphericus*, *Bacillus cereus* (Sudhakar et al. 2008), *Brevibaccillus borstelensis* (Hadad et al. 2005), *Kocuria palustris*, *Bacillus pumilus*, *Bacillus subtilis* (Harshvardhan and Jha 2013), *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas syringae* (Kyaw et al. 2012), *Aspergillus nomius*, *Streptomyces* sp. (Abraham et al. 2017), *Aspergillus glaucus*, *Aspergillus niger*, *Staphylococcus* sp., *Moraxella* sp., *Micrococcus* sp., *Streptococcus* sp. (Kathiresan 2003), *Acinetobacter baumanii* (Pramila and Ramesh 2015), *Penicillium simplicissimum* (Yamada-onodera et al. 2001), *Penicillium pinophilum* (Volke-Sepúlveda et al. 2002), *Stenotrophomonas pavanii* (Mehmood et al. 2016), and *Bacillus amyloliquefaciens* (Novotný et al. 2018). Microbial degradation studies have demonstrated that several bacterial and fungal isolates are capable of degrading PE, but degradation rates vary and are typically low even after extended treatment periods (Restrepo-Flórez et al. 2014).

Waxworms are recent interest for plastic degradation. Caterpillars of the waxmoth *Galleria mellonella* and Indian meal moth *Plodia interpunctella* have been reported for PE biodegradation (Bombelli et al. 2017; Yang et al. 2014). Similarly, the larva of the yellow mealworm *Tenebrio molitor* has been reported to degrade polystyrene and polyethylene (Yang et al. 2015a, b; Brandon et al. 2018). To determine whether the plastic is susceptible to biodegradation and possible depolymerization with the related species, we evaluated the PE degradation capability of the lesser waxworm (*Achroia grisella*). The lesser waxworm is a noxious pest of honey waxcomb, common in most parts of the world. It lives as a nest parasite in the bee colonies and feed on the honey waxcomb (Chandel et al. 2003). Lesser waxworms and greater waxworms are extensively reared and used as an ideal and profitable animal feed for pets, reptiles, and amphibians and also serves as excellent fishing bait. Morphological differences between greater and lesser waxworms are given in supporting information (Fig. S1a and S1b).

The present study reports enhanced biodegradation of PE by the lesser waxworm (*A. grisella*) and also reports the ability of the PE-fed lesser waxworm to complete its life cycle. The egested frass of the lesser waxworm fed on wax comb (WC), PE, and co-feed (PE-WC) were studied by analyzing the changes in physiochemical properties through FTIR and ^{1}H NMR. To the best of our knowledge, there are no previous reports proving the biodegradation of PE by *A. grisella*. The findings of the study indicate that *A. grisella* efficiently degrades PE, and PE degradation rates are significantly enhanced by supplementing the feed with WC. We establish that lesser waxworms fed on such a diet can reproduce a second-generation lesser waxworm which is capable of PE degradation at par with its first-generation counterparts.

Materials and methods

Materials

A. grisella larvae and honeycomb wax were collected from hives of *Apis cerana indica*, apiary farm, Nilambur, Kerala, India (11° 17′ 0″ N, 76° 15′ 0″ E). High-density polyethylene packaging film having a thickness above 51 μm and a density of 0.941 g/m^3 was purchased from the local market, Pondicherry, India. KBr and chloroform-D (purity 99.8%) were purchased from Merck.

Specimen collections and identifications

Based on morphology, the collected larva was identified and validated using available monographs (Ellis et al. 2013; Mahgoub et al. 2015; Sak 2018). The genetic identity of larvae was further confirmed through mitochondrial cytochrome oxidase (mtCOI) gene sequencing studies.

DNA sequencing analysis

DNA (genomic) was extracted from ethanol-preserved specimens using standard extraction techniques (PCBS, Pondicherry) and stored at − 20 °C until use. The specimen

was lysed in the lysis buffer at 50 °C for 3 h. DNA was precipitated, centrifuged from the lysate extract, which was used as unpurified polymerase chain reaction (PCR) templates. The mitochondrial cytochrome c oxidase subunit 1 (cox1) of specimen was used as target genes for PCR primers (COI FP and COI RP) (Robertson et al. 2017). The PCR sequencing was carried out in a 25-μl reaction solution including 1.5 μl each primer for 30 cycles. Purification of resulting PCR products was performed by using the Montage PCR Clean up kit (Millipore). PCR sequencing reaction amplicons were read using a ABI PRISM® BigDyeTM terminator cycle sequencing kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). For the sequencing primer, one of the PCR primers (COI FP and COI RP) was used to determine 26 bases in length of cox1. The COI sequence was blast using the NCBI blast similarity search tool. The phylogenetic trees were constructed using the program PhyML 3.0 aLRT for phylogeny analysis and HKY85 as substitution model. The program Tree Dyn 198.3 was used for tree rendering (Dereeper et al. 2008).

Biodegradation of PE

To assess PE degradation, lesser waxworms were left in contact with the pre-weighed PE film in a 1000-ml glass container. Each container was seeded with 100 lesser waxworms (average weight of 52.2 ± 3.6 mg/worm) plus 2.5 g of PE film. Controls were fed with WC. The weight loss of WC and PE film as a function of time caused by lesser waxworm consumption was measured periodically. For the accurate weight measurement, PE film was washed with 2% (*v*/*v*) sodium dodecyl sulfate (SDS) solution followed with deionized water for several times and were dried for 3 h at 40 °C and weight loss percentage was calculated by using the following formula (Das and Kumar 2015):

$$\text{Weight loss}\ (\%) = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad (1)$$

To check the number of holes formed on the PE surface by lesser waxworm consumption, individual waxworms were left in direct contact with PE film and the number of holes produced was counted at different time interval.

Growth and survivability of PE-consumed lesser waxworms

To assess the growth and survivability of lesser waxworm fed solely on a PE diet, lesser waxworms (100 numbers) with WC, and PE were taken separately in clean glass containers. Waxworms were daily monitored, counted, and dead larvae if found were removed from the container to avoid re-counting. The survival rates and average weight of the lesser waxworm fed on PE samples were calculated in comparison to its conventional diet (WC) under ambient conditions with triplicates for 28 days. To indicate the weight difference in worm biomass before and after PE feeding, the average weight of lesser waxworms that were added to the container was measured using an analytical balance with 0.01 mgs readability and the measurement continued once in 4-days' interval for 28 days of experiment. For the initial 6 days, 2.5 g of WC was added to the container along with PE feed to support the growth of the infant worm but thereafter fed with PE alone. Survival rate (SR) was assessed as the percentage of live lesser waxworms based on the initial number of live lesser waxworms (100 numbers) (Yang et al. 2018). To evaluate the ability of *A. grisella* to complete its developmental stages (larvae, pupa, and adult moth) and also to observe the effect of WC and PE feed on the life cycle longevity of the lesser waxworms, separate experiment was conducted. Lesser waxworms were provided with 5 g of WC and PE. Every 5 days, 2.5 g of WC feed was added to the control container. The duration for the formation of larvae, pupae, and adult moth was observed regularly on each day. Folded paper was added to the larval container after the worm reached the last instar stage. The mature worms migrated to this folded paper to spin their cocoons. These cocoons were collected and transferred to a clean container where the pupae developed into moths. The larval stage is the only developmental stage where the waxworm prefers to eat; therefore, no more WC or PE was added to the pupal container. Adult moth longevity was calculated following its death.

Effects of added nutrition on PE degradation and survivability rate

The effects of added nutrition on PE degradation and survivability rate were studied. The lesser waxworms were fed with PE film (2.5 g) alone and PE-WC co-feed (2.5 g PE and 2.5 g WC) for a period of 28 days. Controls were provided with WC. Each experiment was conducted with 100 lesser waxworms. The lesser waxworms prefer to feed more on WC over PE when unlimited amount of WC is provided. To avoid this, the mass ratio of WC and PE is maintained at a 1:1 ratio (*w*/*w*) throughout the experiment. Every 5 days, the PE-WC co-feed was provided with additional WC to maintain a 1:1 ratio (*w*/*w*) of PE to WC. The weight loss of PE films as a function of time caused by lesser waxworm consumption was measured periodically as described above. Survivability rates of WC, PE, and PE-WC consumed lesser waxworm were calculated as the percentage of live lesser waxworms based on the initial number of live lesser waxworms (100 numbers) (Yang et al. 2018).

Collection and characterization of egested frass

Almost 100 actively feeding third instar stage lesser waxworms were fed with its natural diet WC as control and PE film and PE-WC co-feed, as their sole diet for 16 days. The egested frass of WC, PE, and PE-WC co-fed worms were subsequently collected every 12 h to avoid carryover of un-ingested feed mixing with the accumulated frass. The frass samples were collected, weighed, and stored in airtight container at 4 °C for further analysis.

The residual PE in the egested frass was extracted based on the previously established methods by Brandon et al. (2018). To estimate the mass of residual polymer in the frass, 30 mg of frass sample was transferred to a clean glass vial and extracted with 2 ml chloroform-D (purity 99.8%) by heating at temperatures between 105 and 115 °C for 30 min followed by vortex mixing. The sample solvent mixtures were filtered and transferred into a clean glass vial. The residual polymer in the filtered solution was concentrated by rotary evaporation and the residue obtained was weighed to determine the extractable fraction, a measure of residual non-degraded and partially degraded PE in the frass. Followed by this, the mass balance of PE-fed lesser waxworms was conducted by measuring the weight of WC or PE added to the container, weight of frass before extraction, weight of extractable fraction in frass, and the weight of accumulated biomass.

Fourier transform infrared spectroscopy (FTIR) (Thermo Nicolet; model 6700) was used to confirm the biodegradation of WC and PE films by analyzing egested frass of the lesser waxworm. The control and frass samples were subjected to FTIR analysis by making pellets with KBr and the measurements were carried out between the ranges from 400 to 4000 cm^{-1}.

To apprehend changes in the end products of the egested PE, liquid-state ^{1}H NMR analysis was conducted on a 400-MHz Fourier transform-nuclear magnetic resonance (FT NMR) spectrometer (Avance-II, Bruker). The WC and PE were taken as control and frass of the lesser waxworm fed on WC, PE, and PE-WC was considered as test samples. Each sample (30 mg) was extracted with 2 ml chloroform-D (purity 99.8%) by heating at temperatures between 105 and 115 °C for 30 min followed by vortex mixing for 30 min (Bugada and Rudin 1987). The sample solvent mixtures were filtered before transferring to the NMR tubes. Approximately 1 ml of filtered extract was transferred into 5 mm NMR sample tubes (Sigma-Aldrich) and ^{1}H NMR spectra were measured on a 400-MHz FT NMR spectrometer.

Growth and survivability of second generation of PE-consumed lesser waxworms

A second generation of lesser waxworm fed solely on a PE diet and PE-WC co-feed was reared in the laboratory at ambient conditions in glass containers. Lesser waxworms were fed with PE and PE-WC until pupation. Crumpled paper was added to the larval container after the first mature larvae begin to spin cocoon. The late instar stage larvae migrated to this crumpled paper to spin their cocoon. Some of the larvae spin their cocoon on the PE film surface itself. These cocoons were collected and transferred to a clean container where the pupae developed into moths. Male and female moths were placed in a container to mate. Since female moths prefer surfaces with cracks and crevices for egg laying, folded wax paper was added to the container as a surface for egg laying. The eggs were collected and transferred to a container with excess PE supplemented with waxcomb to support hatchling development. Hatchlings were raised on PE-WC feed until the larvae reached the third instar stage to be transferred into other vessels to test for growth and PE degradation. Experiments to assess PE degradation, survivability rate, and life cycle longevity of second-generation waxworms fed on PE and PE-WC were performed as described above.

Results and discussion

Genomic analysis

The PCR amplified DNA and endonucleases restrictions were applied for interspecies variation (Guillot et al. 2000). The BLAST search of the cox1 sequences of the lesser waxworm showed very close similarities with the *Achroia* of the Pyralidae family, and phylogenetic trees were constructed using program PhyML 3.0 aLRT. The larvae were identified based on molecular taxonomy and phylogeny as *A. grisella*. Nucleotide sequencing data of the lesser waxworm of this study was submitted to NCBI and the accession number MH192348 was obtained. The maximum likelihood phylogenetic tree of *A. grisella* has been shown in Fig. 1 (Dunshea et al. 2008; Nakao et al. 2017). The resultant DNA sequencing of *A. grisella* and phylogenetic trees constructed using different statistical methods are presented in supplementary data file (Figs. S2, S3, and S4).

Biodegradation of PE

PE degradation tests were performed with the lesser waxworm for 8 days. The lesser waxworms were able to chew and make holes in the PE film, when it was left in direct contact with the worm (Fig. 2a, b). Holes started to appear after 45 min with an estimated 2.01 ± 0.97 holes per worm per hour (Table 1). Lesser waxworms feeding activity resulted in a decrease in the weight of WC and PE. The control samples (WC) and PE films were collected to determine weight loss after biodegradation for a comparative study in ambient conditions. The weight of the SDS-washed film reduced due to polymer integrity loss as

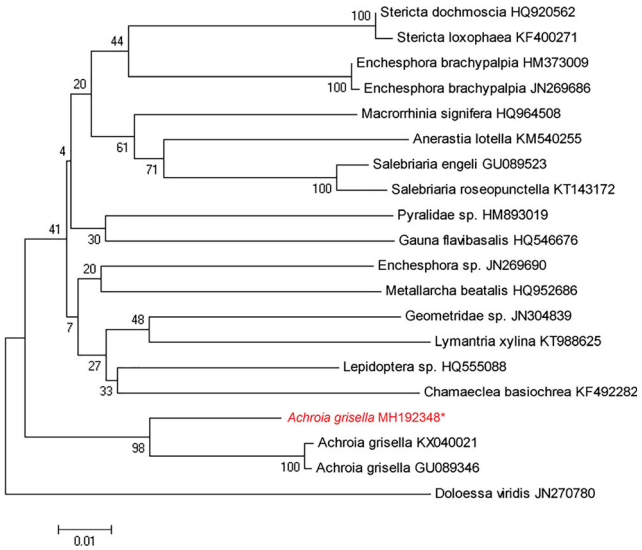

Fig. 1 Maximum likelihood phylogenetic tree for *Achroia grisella* (MH192348)

Table 1 Quantitative estimation of number of holes formed on the PE film surface after exposure to the lesser waxworm (each line in the table represents a separate experiment)

No of worms	No of holes	Time (h)	Hole worm^{-1} h^{-1}
1	1	1	1.00
1	3	2	1.50
1	7	4	1.75
1	13	4	3.25
1	9	4	2.25
1	7	3	2.33
1	11	3	3.67
1	26	24	1.08
1	29	24	1.21
1	47	48	0.98
8	73	24	3.04
		Average	2.01
		St.dv.	0.97

shown in Fig. 2c. The post-degradation weight loss percentage for WC and PE films by a group of 100 lesser waxworms (*A. grisella*) was calculated to be 90.5 ± 1.2% and 43.3 ± 1.6%, respectively. Consumption of PE increased throughout the experiment (Fig. 2d). From the initial 2.5 g PE, the total weight loss at the end of the experiment was 1.46 ± 0.02 g by lesser waxworm fed PE (Supplementary Table 1). Total weight loss of PE accounting for 43.3 ± 1.6% of the initial weight (2.5 g) within 8 days corresponds to an average degradation of 1.83 mg of PE per day per larvae. Recent report on PE degradation with *Galleria mellonella* stated an average PE degradation rate of 0.23 mg cm^{-2} h^{-1} (Bombelli et al. 2017). Like polyethylene, honey comb wax is also a polymer, consisting off long chain carbon atoms bonded together, with other atoms attached to the sides of the carbon chain. *A. grisella* larvae found in nature are evolved to eat and digest beeswax, which has strong chemical bonds similar to PE. The larvae of the Indian meal moth are reported to house PE-degrading bacteria in its digestive tract (Yang et al. 2014). The plastic digesting ability of *A. grisella* larvae might be due to the presence of PE-degrading gut bacteria or any other unique extracellular enzymes which are yet to be pondered.

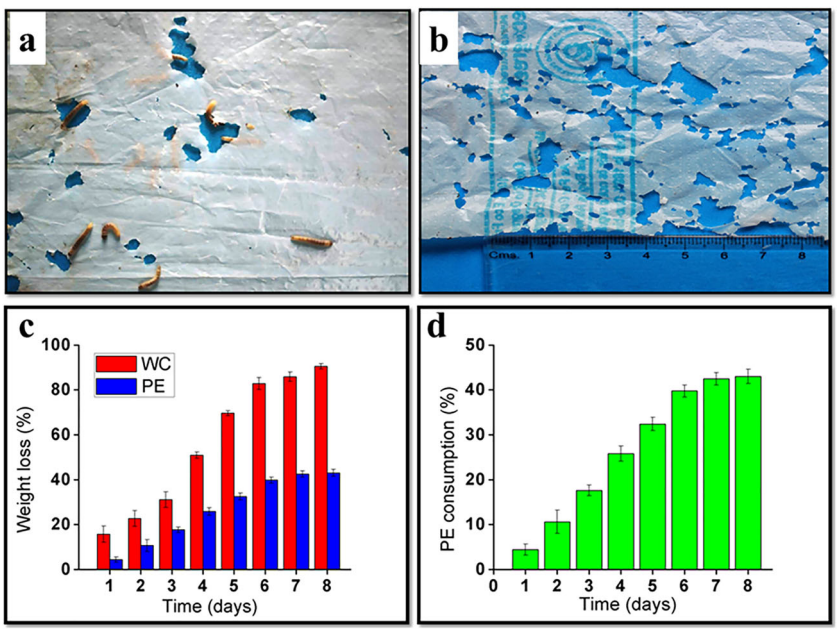

Fig. 2 **a** *Achroia grisella* larvae feeding on PE film. **b** Degraded PE film with holes after exposure to the lesser waxworm for 12 h. **c** Comparison of post-degradation weight loss percentage of waxcomb (WC) and polyethylene film (PE) after lesser waxworm consumption **d** PE consumption (%) over time

Growth and survivability of PE-consumed lesser waxworms

Growth of lesser waxworms fed on WC and PE was observed. The waxworms completed their growth and underwent all developmental stages (larvae, pupa, and adult moth). Lesser waxworms fed with WC and PE completed its life cycle in 43.8 ± 4.7 and 32.2 ± 3.2 days, respectively. Significant differences were observed in the larval duration of WC- and PE-fed worms (Fig. 3a, supplementary Table 2). Compared to PE-fed worms (14.5 ± 2.1 days), WC-fed worms had longer larval stage duration of 25.3 ± 5.1 days. Studies on the life cycle of insects confirmed that the larval stage duration longevity depends on the environmental conditions in addition to the availability and type of food (Mohammadi et al. 2010). Lesser waxworms fed with PE gained less biomass over the course of the experiment than waxworms fed with WC (Table 2). Waxcomb-fed larvae had an increased larval weight of 146.1 ± 2.7 mg per larvae from the initial weight of 7.8 ± 3.6 mg over a period of 28 days. The PE-fed lesser waxworms did not appear to increase their biomass to the same extent as the WC-fed worms. Initial weight gain up to 8 days was more for PE-fed larvae compared to the last 8 days as this container was provided with waxcomb for the initial 6 days to support the juvenile larval growth. Polyethylene-fed larvae attained 73 ± 2.4 mg average weight per larvae from the 6.2 ± 3.4 mg initial weight over a 16-day period. As WC is the conventional food for the worms, they prefer to feed more on WC, and it may be the reason for the increased larval duration than PE-fed worms which in turn caused increased weight gain of the worms. Moreover, PE, unlike WC, does not have the necessary nutrients and proper water content required for growth and development. It may also be the reason for the less biomass gain. At the end of the experiment, the survival rate (SR) for the lesser waxworm fed WC was 91.6 ± 1.01% whereas SR for the lesser waxworm fed PE was 79.7 ± 2.9% (Fig. 3b). It shows that the lesser waxworm fed PE alone can survive by eating and digesting PE but survival rates are low compared to WC-fed lesser waxworms. Dead lesser waxworm counts were observed, and it was found that the PE-fed worms showed a decreased number of dead worms, which indicates that waxworm fed PE alone scavenged essential nutrients needed for growth and survival by consuming dead waxworms. So it can be stated that the reduced SR of PE-fed worms compared to WC-fed worms is because of the occurrence of cannibalism observed between the larvae when there is shortage of food or essential nutrients needed for development (Mahgoub et al. 2015).

Table 2 The effect of WC and PE feed on larval weight of *Achroia grisella*

Time (days)	Average weight of one larvae (mg)	
	WC feed	PE feed
0	7.8 ± 3.6	6.2 ± 3.4
4	21.6 ± 3.5	28.4 ± 3.2
8	56.4 ± 4.0	52 ± 1.1
12	72.1 ± 1.3	65 ± 1.8
16	94.0 ± 4.0	73 ± 2.4
20	120.2 ± 4.1	–
24	132.0 ± 3.0	–
28	146.1 ± 2.7	–

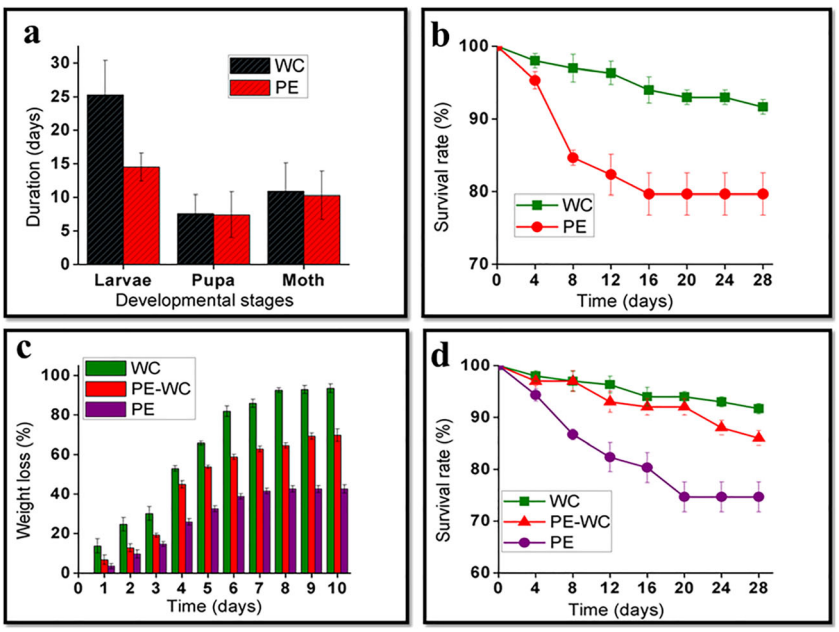

Fig. 3 **a** Time taken to complete different development stages by lesser waxworms fed with WC and PE. **b** Comparison of survival rates of lesser waxworms fed on WC and PE. **c** Effects of added nutrition on PE degradation: post-degradation weight loss percentage for lesser waxworm fed waxcomb (WC), polyethylene film (PE), and PE-WC co-feed (PE-WC). **d** Comparison of survival rates of lesser waxworms fed on WC, PE, and PE-WC

Effects of added nutrition on PE degradation and survivability rate

Addition of a conventional nutrient source enhances biodegradation of PE and other plastics like PS (Brandon et al. 2018; Yang et al. 2018). Waxcomb is the normal feed for lesser waxworms. Waxcomb is made up of beeswax, a mixture of several chemical compounds consisting mainly of esters of various long-chain alcohols and fatty acids by honey bees to raise their larvae and to store honey and pollen. When lesser waxworms were co-fed PE in the presence of WC, they preferred WC over PE. Supplementing the diet of lesser waxworms with WC enabled faster rates of PE degradation compared to PE alone (Fig. 3c). The post-degradation weight loss (%) was 42.5 ± 2.1% for PE alone and 69.6 ± 3.2% for PE-WC co-fed worms. As shown in Fig. 3d, SR was higher for lesser waxworms fed WC (91.3 ± 1.01%) and PE-WC (86 ± 1.4%) than the PE alone (74.6 ± 2.9%). Provision of added nutrition enabled faster PE degradation and higher survivability in PE-WC-fed worms. Both biodegradation and growth require synthesis of enzymes using proper nutrients. It is possible that the diet with added nutrition may facilitate essential nutrients and minerals like calcium, potassium, nitrogen, phosphorus, etc., needed for growth and development of the larvae other than the hydrocarbon present in the PE enabling increased SR of the worms.

Characterization of egested frass

The egested frass of worms contained an extractable fraction of residual PE and/or modified PE after digestion. The residual PE was extracted from the egested frass of PE, PE-WC fed lesser waxworms using chloroform-D and estimated mass balance of PE-fed lesser waxworms. The extractable fraction/residual polymer of frass from lesser waxworms fed on PE, PE-WC decreased over the course of the 16-day experiment. The percentage of non-degraded/partially degraded PE in the frass decreased from 62 ± 1.0% on day 4 to 31.2 ± 1.01% by day 16 (Fig. 4a).

A perfect mass balance was not obtained in the experiment; it exhibited deviation which may be due to the losses into the gas phase H_2O, CO_2 (Brandon et al. 2018). Deviation in mass balance increased from day 4 to day 16. For lesser waxworms fed on PE, the deviation in mass balance was 55.4 ± 2.01% whereas for PE-WC-fed worms it was 25 ± 2.01% at the end of the 16-day experiment (Fig. 4c, d) which may be due to the increased biomass accumulation in PE-WC-fed worms. A decrease in the residual PE and increased deviation in mass balance over the course of the experiment for waxworm-consumed PE indicated that most of the ingested PE was either degraded or was incorporated into waxworm biomass.

The egested frass of lesser waxworm fed on PE was extracted to quantify the biodegradation of polymer hydrocarbons. Biodegradation of PE through the chemical modifications in the waxworm gut was studied via FTIR and ^{1}H NMR techniques. The FTIR analysis is one of the important proofs to quantify the efficient bio-mineralization by degradation/change in the intensity of native bonds which appeared in PE (Mamoor et al. 2011). While PE and WC act as control and positive control for the degradation analysis, FTIR spectrum of control PE and positive control (WC) (Fig. 5) shows the peaks corresponding to the C-H rocking alkanes (725–720 cm^{-1}), C-N stretching aliphatic amines (1173–1020 cm^{-1}), N-O asymmetric stretching

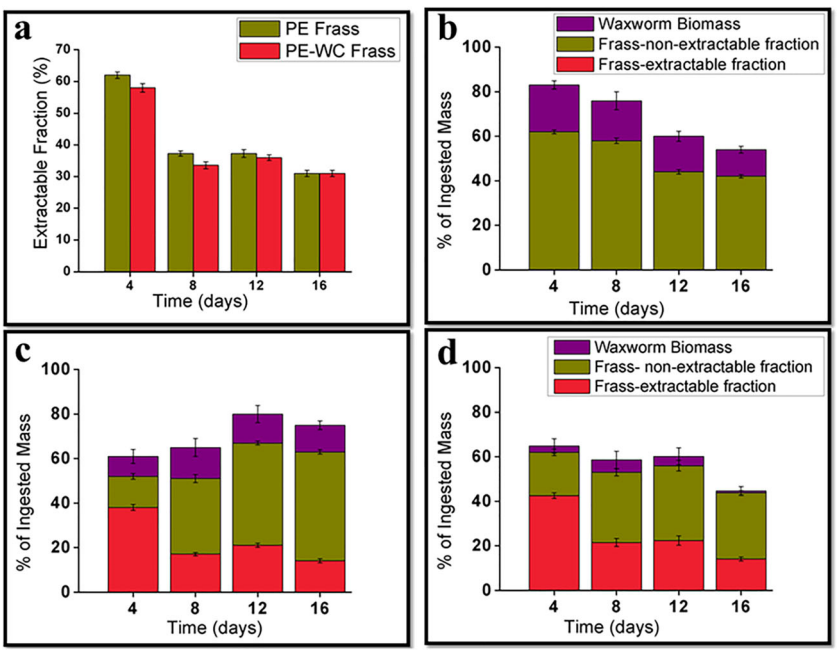

Fig. 4 **a** Changes in the extractable fraction of the frass of PE and PE-WC fed worms. **b** Mass balance of lesser waxworms fed on WC, **c** PE-WC, and **d** PE over a 16-day experiment

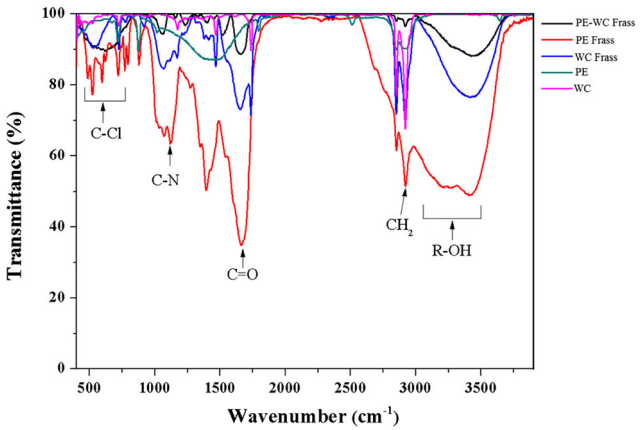

Fig. 5 FTIR spectra of control and frass samples for lesser waxworms fed waxcomb (WC), polyethylene (PE), and PE-WC co-feed (PE-WC)

nitro compounds (1477 cm^{-1} in PE), C-H bending alkanes (1467 cm^{-1} in WC), the C-O and C=O stretching in the ester functional group (1796–1736 cm^{-1}), and C-H asymmetric and symmetric stretching alkanes group (3000–2850 cm^{-1}) (Shearer 1989; Gulmine et al. 2002; Ibiene et al. 2013; Ingavale and Raut 2018). The PE frass samples from lesser waxworms showed the formation of a new O-H stretching alcohols/phenols group absorbance peak (3500–3200 cm^{-1}) by decreasing the growth of strong intensity of the CH_2 group might be due to the oxidation in the gut of lesser waxworms, which was observed during PE degradation tests by fungal and bacterial cultures (Asgari et al. 2014; Mukherjee et al. 2018). Significant disappearance of absorption band at 1796 cm^{-1} in the frass of PE and raising of a new carbonyl group peak with broadening the intensity was observed which might be oxidation of PE by overlapping of various carbonyl compounds with amides (1660–1654 cm^{-1}) (Khabbaz et al. 1999; Xu et al. 2018; Muhonja et al. 2018). The peaks around 3400 and 1654 cm^{-1} were assigned to protein material which may due to the enzymatic action of gut bacteria (Bonhomme et al. 2003). The FTIR analysis of frass samples revealed the increase in the intensity growth of C–N stretch aliphatic amines (1000–1200 cm^{-1}) and the other peaks assigned to C-Cl stretch alkyl halides (850–550 cm^{-1}). While the lesser waxworm fed on PE mixed with natural diet WC shows new peaks of amides II and III due to stretching vibrations of N-H asymmetric nitro compounds and plane vibrations of C-N stretch aliphatic amines and -C(triple bond) C-H:C-H bend alkynes (1520, 1223, and 623 cm^{-1}) (Silva et al. 2008). The FTIR results illustrate the biodegradation of PE, and WC as frass of all the samples in comparison with control samples (PE, WC) revealed reduction, shift, and appearance of new peaks.

Table 3 Carbonyl index from FTIR spectra peaks of PE to frass of PE and frass of PE-WC

S. no	Polyethylene	C.I
1.	Control (PE)	0.005 ± 0.01
2.	PE Frass	0.01 ± 0.01
3.	PE-WC Frass	0.007 ± 0.04

CI values are means ± S.D. ($n = 3$)

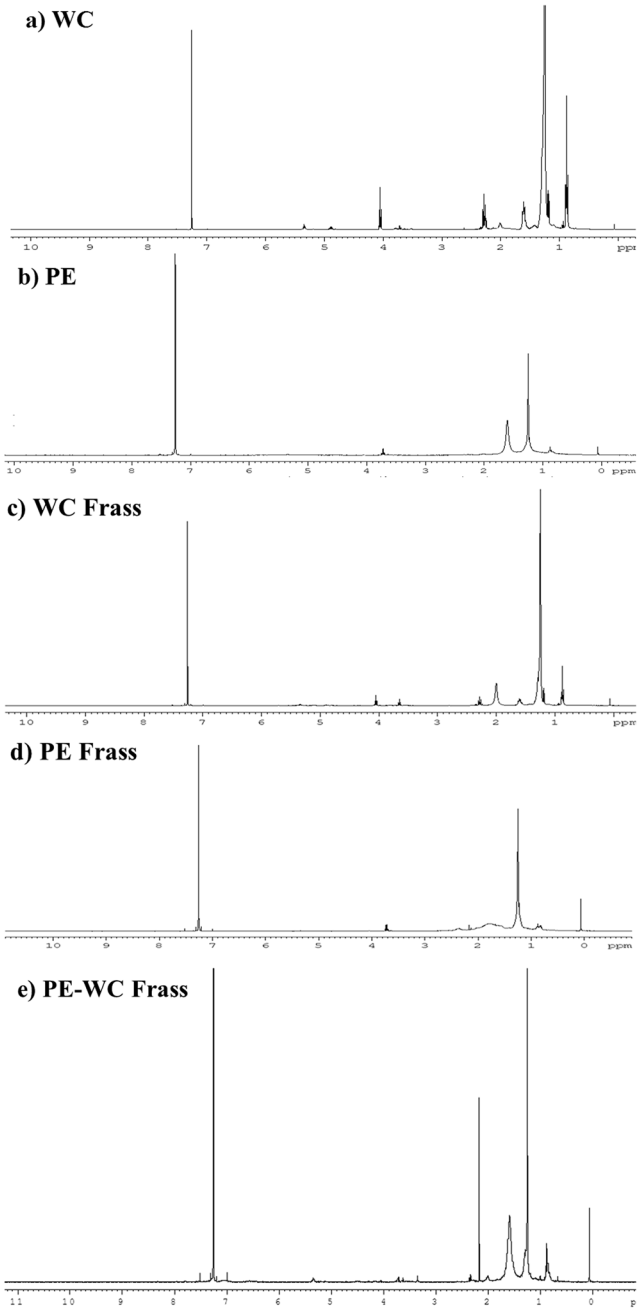

Fig. 6 ^{1}HMR spectra of control and frass samples for lesser waxworms fed on WC, PE, PE-WC cofeed (**a**) waxcomb (WC) (**b**) Polyethylene (PE) (**c**) Waxcomb frass (WC frass) (**d**) Polyethylene frass (PE frass) (**e**) PEWC cofeed Frass (PE-WC frass)

The biodegradation of PE was estimated using the carbonyl index, which is defined as the ratio between the area of absorbance peak of carbonyl groups band (1650–1700 cm^{-1}) to that area of CH_2 scissoring peak bond (around 718 cm^{-1})

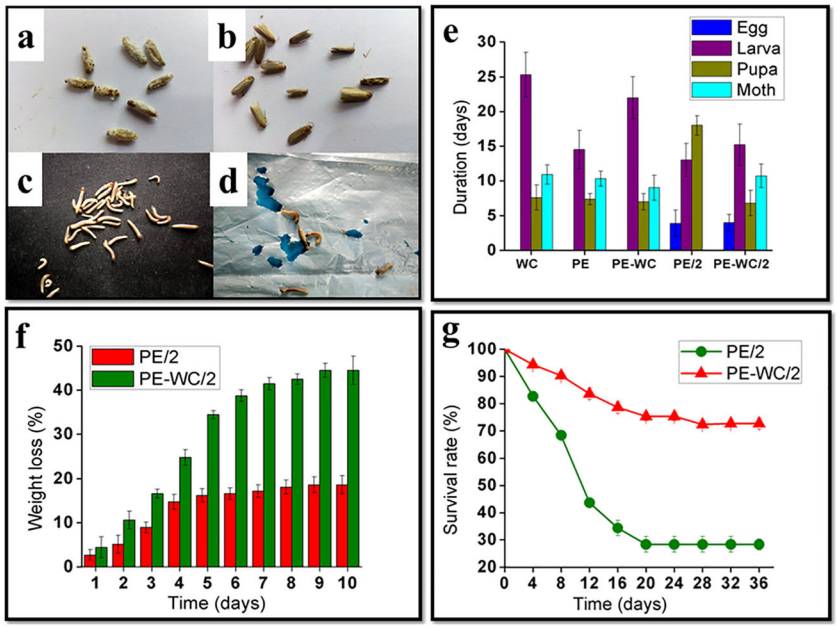

Fig. 7 PE degradation by second-generation *A.grisella* larvae fed solely on PE (PE/2) and PE-WC co-feed (PE-WC/2). **a**–**d** Life cycle stages for a second-generation PE-WC fed lesser waxworms, showing **a** pupae, **b** adult waxmoth, **c** infant waxworms, and **d** PE eating second generation. **e** Comparison of developmental stage duration of first- and second-generation lesser waxworms fed on waxcomb (WC), polythene (PE), and polythene-waxcomb co-feed (PE-WC); here, WC, PE, and PE-WC denotes first-generation lesser waxworms and PE/2 and PE-WC/2 denotes second-generation lesser waxworms. **f** Post-degradation weight loss percentage of PE and **g** survival rate percentage for second-generation lesser waxworms

(Andrady et al. 1993; Hadad et al. 2005). The PE frass results showed that the biodegradation through the lesser waxworm reduced the CI by 80% followed by PE-WC frass than control PE (Table 3). Apparently, it is evident from the analysis that the waxworm gut/gut flora might release the extracellular enzymes, which are actively involved in the degradation of PE.

Additional confirmation of PE biodegradation through chemical modifications in the egested frass of the lesser waxworm was attained by the ^{1}H NMR technique. In the ^{1}H NMR analysis, the control sample (Fig. 6a, b) shows two different blocks strongly for CH_3 and CH_2 attributed around 0.87–1.25 and 3.7 ppm. The peak at 1.6 ppm might be due to the allylic hydrogen to the carboxylic C=O group. The frass of PE and PE-WC samples (Fig. 6d, e) shows a chemical shift in various organic compounds in the 0.8- to 2.3-ppm region. Based on the change in shift of the peaks, the organic species possible considered as: aliphatic protons from the $\delta_H = 0.8$ to 3.7 ppm region (H-C, CH_3, and CH_2); shift to α position carbon atoms to Cα-C=O or C=N group (carbonyl/aminoic), Cα-C-H groups (unsaturated allylic). The peak at 1.2 ppm corresponds to lipids and 1.7 ppm to different amino acids (Kutyshenko et al. 2015; Eyheraguibel et al. 2017; Paço et al. 2017). Comparatively, the intensity of peaks in frass samples was increased in the different regions around 1.1 to 2.3 ppm. The ^{1}H NMR results were consistent with the new carbonyl and alcoholic groups in FTIR analysis.

PE degradation by second generation of lesser waxworms

Lesser waxworms fed solely on a PE diet and PE-WC feed completed their life cycle, developing into pupae then moth in 32.2 ± 3.2 and 38.1 ± 2.2 days, respectively (supplementary Table. 2). Eggs laid by the moths hatched with an average duration of 4 days (Fig. 7e). A new generation of lesser waxworms was then reared in the laboratory at ambient conditions for 6 days, with excess PE supplemented with waxcomb to support hatchling development. This second-generation lesser waxworms were then fed with 2.5 g of PE film for 10 days (100 worms with an average weight of 52.2 ± 2.6 mg per worm). PE consumption percentage and survival rates for the second-generation PE-WC-fed lesser waxworms (PE-WC/2) were comparable to those of the first generation (Fig. 7f, g) with a PE consumption rate of 44.9 ± 3.2% and SR of 72.6 ± 2.01%. The second generation of PE-fed worms (PE/2) had a PE consumption rate of 18.6 ± 2.1% and SR of 28.3 ± 2.01%. The second generation of PE diet had high initial mortality and newly hatched worms failed to establish themselves. Some of the worms formed cocoons following the larval phase but did not develop into moth. The second-generation lesser waxworms of PE-WC eventually completed their life cycle, evolved to be mature larvae, and then developed into pupae (6.8 ± 1.8 days) and moth (10.7 ± 1.7 days). These results indicate that the feed supplemented with

additional nutrition (WC) enhances the rate of PE degradation and lesser waxworms which consumed such a diet can develop into the next generation with a favorable PE degradation.

Conclusion

A. grisella larvae ingesting and degrading PE as a sole diet survived for almost 1 month, developing into a second generation, though the PE diet did not provide enough nutrients needed for growth and survival. Provision of added nutrition enhanced PE degradation, allowing high survival rate and enabled breeding of a second generation with favorable PE biodegradation. The FTIR and ^{1}H NMR analyses of egested frass confirmed biodegradation of PE in *A. grisella* larvae as the presence of new carbonyl and alcoholic groups with increase in unsaturated hydrocarbon was observed. Further studies using gel permeation chromatography (GPC) might help to understand the impact of molecular weight of PE material on biodegradation and change in molecular property of PE polymer after it passes through the gut of lesser waxworms. Further research is needed to understand whether the PE biodegradation is gut microbe-dependent, similar to yellow mealworms (Yang et al. 2018), or gut microbe-independent, i.e., basically by digestive enzymes of lesser waxworms. Future work should also concentrate on elucidating the mechanism of degradation within the lesser waxworm to enable viable applications.

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