Frontiers in Bioscience Elite

Front. Biosci. (Elite Ed) **2022**; 15(2): 11
https://doi.org/10.31083/j.fbe1502011

Short Communication

Ingestion of Nylon 11 Polymers by the Mealworm (*Tenebrio molitor*) Beetle and Subsequent Enrichment of Monomer-Metabolizing Bacteria in Fecal Microbiome

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Academic Editor: Mihail Lucian Birsa
Submitted: 8 October 2022 Revised: 1 November 2022 Accepted: 7 November 2022 Published: 5 May 2023

Abstract

Background: Nylon 11 is a synthetic plastic widely used in commercial products such as tubing for automobiles, offshore oilfields, and medical devices. An increasing amount of nylon and other plastic wastes have been released into various environments, posing ecological threats. The biodegradation of bundled nylon polymers has been considered impossible due to their crystalline structures. **Methods**: Nylon 11 film was created and incubated with adult mealworms. The mass, as well as structures, of nylon 11 films at pre- and post-incubation with beetles were compared. The number of nylon 11 monomer degrading bacteria in feces were determined by culture-dependent approach. The *t*-test was utilized to examine the statistical significance. **Results**: We discovered that adult mealworm (*Tenebrio molitor*) beetle can ingest nylon 11 when stretched thin. The microscopic observation of their feces did not identify the presence of large fragments of nylon 11. The analysis of fecal bacteria revealed that while the total number of culturable bacteria did not change significantly, the number of 11-aminoundecanoic acid-metabolizing bacteria increased by 10,000-fold. **Conclusions**: Our results suggest that bundled nylon 11 polymers were fragmented into smaller pieces, including monomeric units (11-aminoundecanoic acid) by adult mealworm. The monomers seem to have supported the proliferation of gut microbial communities capable of utilizing 11-aminoundecanoic acid as a carbon and nitrogen source. Our work implies the potential use of the mealworm beetle as a means to fragment nylon polymers for remediation applications.

Keywords: nylon biodegradation; plastics biodegradation; insect; gut microbiota; mealworm beetle; *Tenebrio molitor*

1. Introduction

Plastics are synthetic polymers with incredible durability and chemical resistance. They are extensively used in industrial practices, as well as in commercial products [1,2]. While recycling programs have been developed, a significant portion of plastic waste is still destined for landfills. Contamination of environments by various plastics are also of significant concern [1,3,4]. There are an increasing number of studies investigating microbial degradation of plastics to address these concerns. However, they are generally limited to amorphous plastics and oligomers [5–10]. The degradation process of crystalline plastics (such as nylon 11) is extremely slow due to their chemical and physical stability, which hinders efficient fragmentation and deterioration [11].

Enhanced fragmentation and/or deterioration accelerates the overall biodegradation rate of plastics [12]. A variety of mechanical, chemical and UV-based mechanisms induce fragmentation of bundled polymers into smaller fragments [13,14]. Several studies demonstrated that invertebrates can serve as agents of such modifications [15–22]. In particular, *Tenebrio molitor* (mealworm) has been demonstrated to decompose a variety of plastics [23,24]. Additionally, the involvement of gut microbiota in further fragmentation and/or metabolic degradation of fragmented pieces (e.g., monomers, dimers, and oligomers) has been demonstrated for some plastics (e.g., polystyrene, polyethylene) [7,25].

Nylon 11 is a synthetic polymer widely used as tubing, textiles and coating for a variety of commercial products [26]. It is synthesized by polymerization of monomeric 11-aminoundecanoic acid (11-AUA). It is considered a bioplastic since 11-AUA is derived from castor oil [27]. Nylon 11 polymers form extensive hydrogen bonding between the polymers, resulting in a crystalline structure [28]. This leads to its strong physical properties, which makes nylon 11 a desirable material for commercial products, but prohibits degradation when they are no longer in use [6]. The possibility of nylon 11 fragmentation in the environment has been suggested [29], however, to the best of our knowledge, a specific organism that is responsible for degradation has not yet been identified. Additionally, invertebrate's ability to ingest and/or biodegrade any nylon polymers (including well-studied nylon 6, nylon 6,6) has not yet been reported. Past investigations have been limited to nylon oligomers.

In this study, we discovered that mealworm beetles gnaw melt-stretched nylon 11 film at the rate of 18 mg per day per gram of beetles. We also detected a 10,000-fold in-

crease in the number of gut bacteria capable of growing on nylon 11 monomer (11-aminoundecanoic acid) as the sole source of carbon and nitrogen. These data suggest the possibility that ingested nylon 11 pieces are fragmented, further processed and metabolized by their gut microbiota. This is the first study demonstrating the ability of insects to fragment nylon plastics as well as possible contributions of microbiota for mineralization.

2. Materials and Methods

2.1 Materials and Organisms

Nylon 11 and other chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Deionized water was used for generating bacterial growth media. DEPC-treated water was used for molecular experiments. Bacterial strains were grown on a modified defined media [30] supplemented with 11-aminoundecanoic acid as a sole carbon and nitrogen source. The media contained 4.25 g·L^{-1} K_2HPO_4, 1.00 g·L^{-1} $NaH_2PO_4{\cdot}H_2O$, 0.20 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 0.012 g·L^{-1} $FeSO_4{\cdot}7H_2O$, 0.003 g·L^{-1} $MnSO_4{\cdot}H_2O$, g·L^{-1}, 0.003 g·L^{-1} $ZnSO_4{\cdot}7H_2O$, g·L^{-1}, 0.001 g·L^{-1} $CoSO_4{\cdot}7H_2O$, and 11-AUA in saturation. Strains were also grown on modified tryptic soy media (TSA) consisting of 4.25 g·L^{-1} tryptone, 0.75 g·L^{-1} soytone, 0.625 g·L^{-1} dextrose, 1.25 g·L^{-1} sodium chloride, and 0.625 g·L^{-1} dipotassium phosphate (DOT scientific Inc., Burton, MI, USA). Nylon 11 was heated, then stretched to approximately 0.1 mm thickness at room temperature [28]. Stretched filaments (in addition to a film) were also obtained in this process and used for visualization. *Tenebrio molitor* was purchased from Ward's Science (Rochester, NY, USA).

2.2 Incubation of Nylon 11 with Tenebrio Molitor Beetles

Approximately 0.45 g of nylon 11 films were added to twenty mealworm beetles and incubated at room temperature. The exact mass of nylon 11 and the larvae were measured at the beginning of the study and after 50 days of incubation. Before each measurement, plastics were rinsed with water and dried completely at an ambient temperature for three days. The photographs of the nylon 11 pieces were recorded by a phase contrast microscope, Olympus BX60, coupled with DP70 (Olympus, Tokyo, Japan). The images of lower magnification were obtained by Olympus CKX41 and recorded via Mic-Fi digital microscope (Italeco S.R.L., Italy).

2.3 Growth Analysis of Gut Bacteria

A sample of 10 mg of feces was resuspended in 1 mL sterile 50 mM phosphate buffer (pH 8.0) and vortexed before serial dilution was performed. Cells at 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated following the method described by Whitmire [31]. Cells were replica plated on TSA and the modified defined media supplemented with 11-aminoundecanoic acid (0.04%) as a sole source of carbon and nitrogen. Cells were incubated at 30 °C for 2 days and 8 days for TSA and defined media, respectively.

2.4 DNA Extraction and Determination of 16S rRNA Sequence

11-AUA-metabolizing bacterial strain was isolated and its DNA was extracted using E.Z.N.A. Genomic DNA isolation kit (Omega Bio-tek Inc., Norcross, GA, USA). 16S rRNA fragment was amplified by polymerase chain reaction (PCR) using the primer 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) [32]. The 16S rRNA sequence was obtained with the Sanger sequencing by Psomagen Inc. (Rockville, MD, USA). Homologous sequences were identified using NCBI BLAST [33]. The phylogenetic tree was constructed by the maximum likelihood method and Tamura-Nei model with bootstrap value of 500 using MEGA [34].

3. Results and Discussion

3.1 Nylon 11 Ingestion by Adult Mealworm Beetles

The nylon 11 that we acquired from Sigma Aldrich Inc. came as solid spheres of approximately 3 mm in diameter (Fig. 1a). Beetles did not masticate the intact nylon 11 beads. We reasoned that this was due to the size and a lack of accessible surfaces. To reduce the thickness of the nylon 11, low heat was applied, and the plastic was stretched thin (Fig. 1b). Extensive mastication of nylon 11 films by beetles was observed (Fig. 1c). Results of mastication were imaged using stretched filament (Fig. 2). The films and filaments retained structural integrity and did not break down in the absence of biological treatment (Fig. 2a). Mastication was localized to the edge of the film/filament, suggesting the preference of beetles to gnaw freely accessible edges (Fig. 2b).

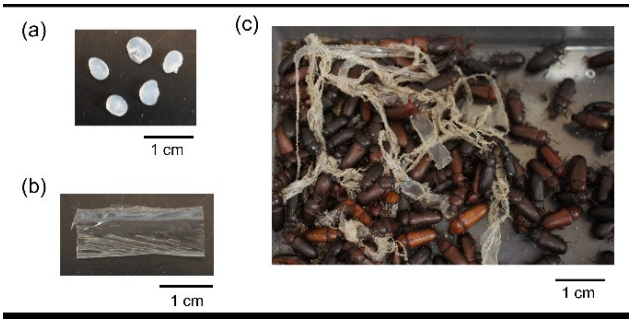

Fig. 1. Nylon 11 pellets, film and films after incubation with adult beetles. (a) Nylon 11 pellets. (b) Stretched nylon 11 film. (c) Nylon 11 films incubated with mealworm beetles. The photograph was taken after 2 days of incubation.

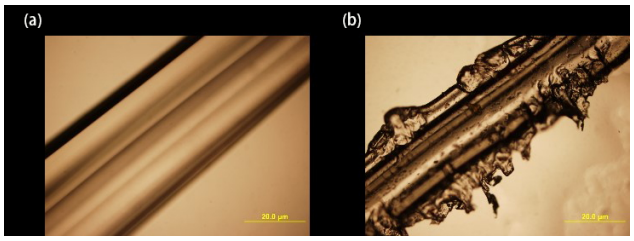

Fig. 2. Microscopic images of nylon 11 filament with and without incubation with beetles. Images were taken (a) without incubation and (b) with incubation with beetles.

To study the rates of mastication, mass of total nylon 11 films before and after incubation with twenty beetles (total body mass 1.990 g) were measured. The mass of nylon 11 film was decreased from 0.441 g to 0.401 g when incubated by twenty beetles. The 0.040 g difference was lost over a 50-day period, which was calculated to be 9.1% of the original nylon mass. The average of three independent trials are shown in Fig. 3a. Because the total mass of nylon and twenty beetles were similar but not identical, the percent loss of mass was graphed.

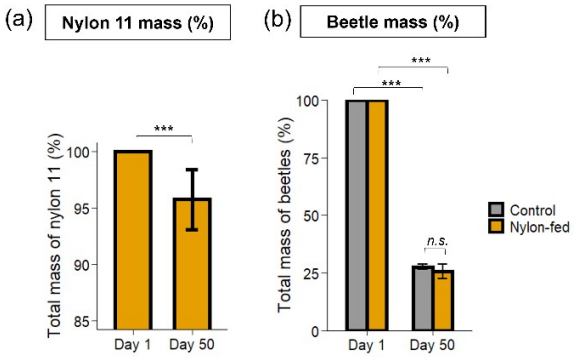

Fig. 3. Changes in nylon 11 and body mass after 50-day incubation. (a) Mass of nylon 11 and (b) the % of initial mass of beetles before and after 50-day incubation. *p*-values were calculated with the Mann-Whitney U test. The asterisk (***) indicates statistical significance (*p*-value < 0.01); n.s., non-significant differences

3.2 No Impact of Nylon 11 Ingestion on Worm's Body Mass

Beetles were incubated with nylon 11 in the absence of any other carbon, energy, and water sources for 50 days. The control groups were left without any organic material and water. Despite a lack of food and water sources, all of the beetles from both groups remained alive for a 50-day period. The mass of beetles at the end of the incubation period did not show a significant difference between nylon-fed and control groups ($25.6 \pm 3.1\%$ and $27.6 \pm 1.0\%$ of original weight, respectively; Fig. 3b). A lack of increase in body mass implies that nylon 11 does not provide sufficient, if any, amount of energy and material to support beetle's growth. The ingestion of plastics that did not result in an increase in the host's biomass has been observed for other plastics as well [16,20,35]. It is of note that nylon 11 ingestion did not decrease the beetles' mass, nor induce early death. Therefore, while it does not provide energy, it also does not seem to negatively impact host's physiology.

3.3 Study of Excrement from Nylon 11 Ingested Worm

To investigate the fate of fragmented nylon 11 in the beetles, we first examined the content of feces under the microscope. There was no detectable difference between feces from nylon 11-fed and control beetles (Fig. 4). However, due to the presence of exoskeletons and other cell-derived macromolecules, we cannot completely rule out the possibility that small, fragmented nylons were excreted. Our attempt to detect nylon 11 fragments with LC-MS did not identify a peak associated with nylon 11 monomer (11-AUA).

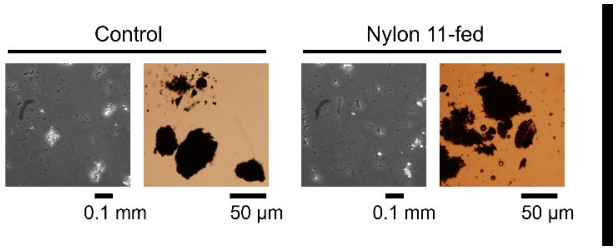

Fig. 4. Fecal matter of beetles with and without nylon 11 feeding. The black marks in the left-side of the lower magnification images are visual artifacts.

Second, we examined whether nylon 11 ingestion changed the gut microbial compositions. Specifically, enrichment of bacteria capable of metabolizing a predicted degradation intermediate (11-AUA) was examined. Gut microbes of nylon-fed and control beetles were compared through spot plating of their fecal matter. On TSA media, there was no statistically significant difference in numbers of colony forming unit (C.F.U.) (*t*-test, *p*-value > 0.05; Figs. 5,6a). C.F.U. per 10 mg of fecal matter were $5.46 \pm 1.72 \times 10^5$ and $3.29 \pm 1.79 \times 10^5$ for nylon-fed and control beetles, respectively. In contrast, when fecal matter was plated on the defined media with 11-AUA as the sole source of carbon and nitrogen, C.F.U. differed significantly between control and nylon 11-fed beetles (Figs. 5,6b). C.F.U. on nylon-fed samples ranged from 100 to 5×10^5 while only 0 or 1 colonies appeared on control samples. The differences in C.F.U. on 11-AUA media were statistically significant (*t*-test, $p < 0.05$). It should be noted that there was variability of C.F.U. among the nylon 11-fed samples, despite that the same supply of worms were used and the experiments were performed simultaneously. The reason for the variability remains unknown.

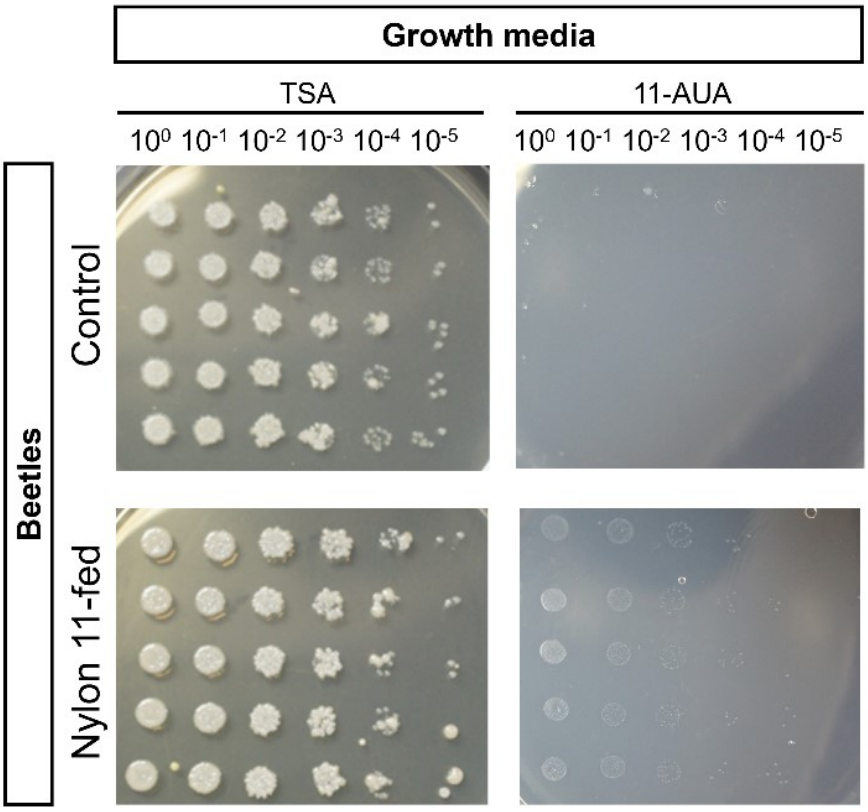

Fig. 5. Growth analysis of bacteria from fecal matter on TSA and defined media with 11-AUA. A fecal matter (10 g) was suspended in 50 mM phosphate buffer and underwent serial dilutions. Solutions are replica plated on TSA and defined media supplemented with 11-AUA as the sole source of carbon and nitrogen.

The ratio of C.F.U. on TSA and 11-AUA media was compared (Fig. 6c). Between 0.03% and 25% (average 4.6%) of culturable gut bacteria in nylon-fed beetles appeared to be capable of metabolizing 11-AUA. This is a 4×10^4 times higher proportion of 11-AUA-metabolizing bacteria than in control (between 0 and 0.0005% with average 0.00011%). A significant increase in 11-AUA degrading bacteria in their gut suggests that (1) nylon 11 was fragmented to monomer (11-AUA) and (2) at least a portion of 11-AUA was metabolized by gut bacteria to increase its abundance. We plan to study whether carbons in 11-AUA are completely mineralized by the gut microbe and/or some metabolites are absorbed by the host in future studies.

3.4 Isolation and Characterization of 11-Aminoundecanoic Acid Degrading Bacteria from Nylon11-Fed Beetles' Excrement

The colony morphology and growth pattern of 11-aminoundecanoic degrading colonies were uniform. The identity of representative 11-AUA degrading strain was analyzed by 16S rRNA sequencing. The sequence showed the highest similarity to those of strains of genus *Serratia*. *Serratia* belongs to class gamma-proteobacteria (Fig. 7). *Serratia* strains are found in a wide range of locations, such as soil, insects, plants and aquatic environments [36]. Some strains are known to degrade plastics or are found in consortium degrading plastics, such as low density polyethylene [37,38], polylactide (PLA) and polyethylene terephthalate (PET) [39].

Plastic-degrading *Serratia* strains are also found in the gut of plastic-fed insects [40]. For example, styrofoam-degrading *Serratia* sp. strain WSW (KCTC 82146) was isolated from the gut flora of larvae of the darkling beetle (*Plesiophthalmus davidis*) [40]. The contribution of resident insect gut microbes in complete mineralization of plastics, especially polyamides, are not well understood. We are currently investigating the enzymes and pathways involved in nylon 11 metabolism.

4. Conclusions

In conclusion, we identified that nylon 11 film can be ingested by the mealworm beetle, *Tenebrio molitor*. The alteration of macroscale structures (i.e., beads vs. film) was crucial for this process to occur. While it does not seem to support beetle's growth in our current condition, the enrichment of nylon 11 monomer degrading bacteria (e.g., *Serratia* spp) in their gut was detected. Our results suggest a model for nylon 11 degradation by *Tenebrio molitor*:

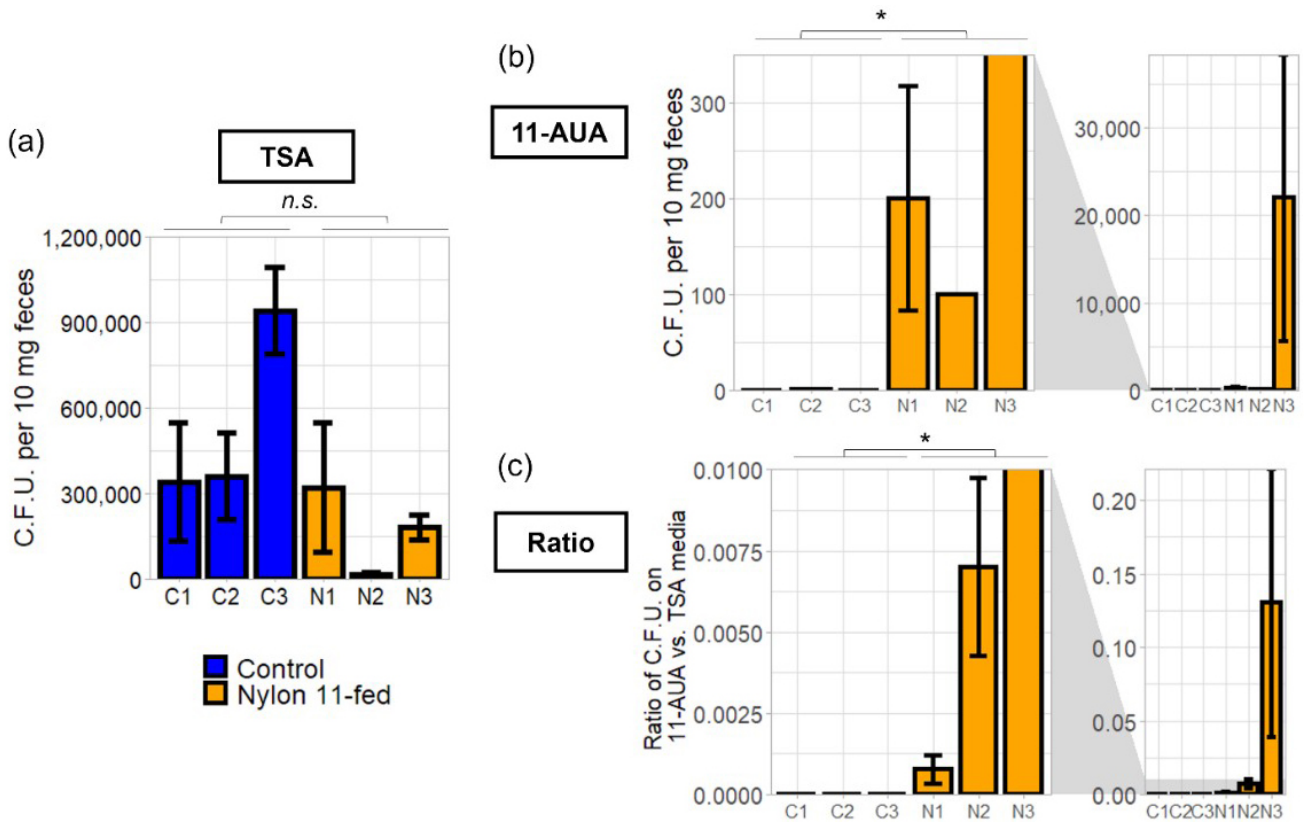

Fig. 6. C.F.U. of fecal bacteria on TSA and 11-AUA media. C.F.U. of 10 mg of feces on (a) TSA and (b) defined media with 11-AUA as sole source of carbon and nitrogen. (c) The ratio of C.F.U. on 11-AUA vs. TSA media. *p*-values were calculated with the Mann-Whitney U test. The asterisk (*) symbol indicates statistical significance (*p*-value < 0.1); n.s., non-significant differences

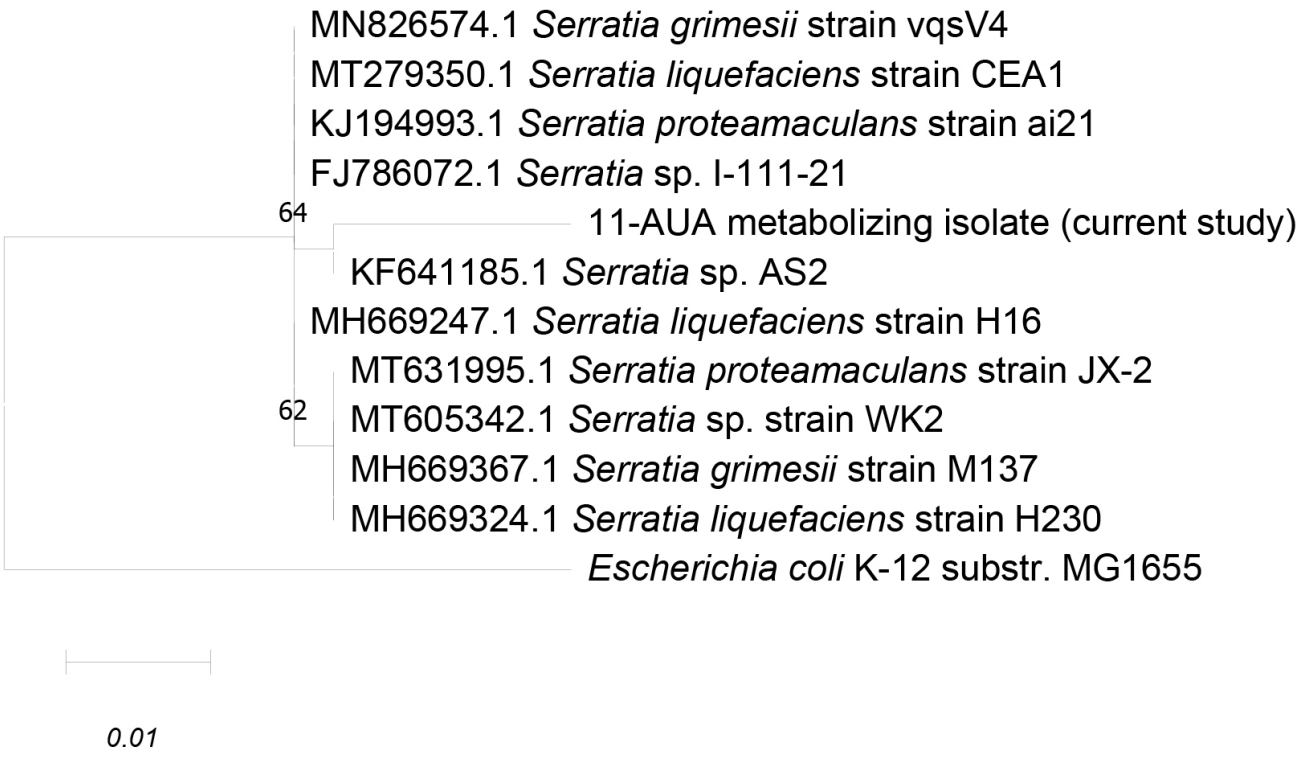

Fig. 7. Phylogenetic tree of the 11-AUA metabolizing bacterial strain isolated from nylon 11 fed beetles. The phylogenetic tree was constructed using 16S rRNA gene sequence. Bootstrap values are shown.

initial fragmentation by mastication, followed by bacterial metabolism in their gut.

Abbreviations

11-AUA, 11-aminoundecanoic acid.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AL and HM designed and performed the research study. HM analyzed the data. HM wrote the manuscript. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The work was funded by the Grants-in-Aid from Indiana University Kokomo Office of Academic Affairs. Authors also acknowledge the support from the Indiana University Kokomo School of Sciences. We thank Peter Tupa for proofreading of the manuscript.

Funding

The study was supported by the Grants-in-Aid from Indiana University Kokomo. This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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