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Polystyrene degradation by bacteria isolated from the larvae of *Rhynchophorus phoenicis*

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Abstract

The larvae of insects of the order Coleoptera have been reported to biodegrade plastics aided by their chewing mouthparts and the activities of their gut biota. However, there is no report of this ability by the African palm weevil (*Rhynchophorus phoenicis*). This study aims to the ability of *R. phoenicis* larvae to biodegrade polystyrene (PS). A total of 100 *R. phoenicis* larvae were fed for 21 days with PS foam, and afterwards, the gut contents of survivors were investigated for possible PS-degrading bacteria. Bacterial isolates were screened for PS biodegradation in an Erlenmeyer flask with PS film as the sole carbon source, in a mineral salt medium (MSM) at a temperature of 30°C and a pH of 7, for a period of 28 days. The isolates were used for biodegradation assay under the same conditions, for 60 days. The weight of PS films was determined before and after the biodegradation assay. Chemical changes in the films were confirmed by Fourier Transform Infrared (FTIR) spectroscopy. Two bacterial isolates were recovered from the gut of the only surviving *R. phoenicis* larvae fed with 100% PS. The isolates were identified based on their 16S rRNA sequences as *Lysinibacillus macriodes* and *Pantoea dispersa* with accession numbers OQ652017 and OQ652023 respectively. The isolates caused an 8.8% reduction in the weight of PS film and FTIR spectroscopy results confirmed the formation of groups suggestive of degradation products with the carbonyl group showing up as absorption peaks in the range of 1640-1760 cm^{-1} and the hydroxylic group at 3000-3700 cm^{-1}. The isolates were able to produce polyhydroxyalkanoate (PHA) equivalent to 1.4g/L, under PS degradation conditions. Therefore, coupling the biodegradation of PS with PHA production could be useful for the valorization of PS waste.

Keywords: *Lysinibacillus macriodes*, *Pantoea dispersa*, Polystyrene, polyhydroxyalkanoate
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Introduction

One hallmark of human evolution is the continual synthesis of xenobiotic compounds for industrial and domestic applications for the advancement of our lives. A notable footprint of human technological advancement has been the drastic transformation of the biosphere composition (Chure et al., 2021). There is no sign of this slowing down as new synthetic products continue to enter the market and find more applications. We are going to live with the problem of plastic pollution for a long time, given their assortment of usage,
which has transformed the entire world of material usage for households, industries and building construction (Wright et al., 2017; Tareen et al., 2022).

Plastics have emerged as a xenobiotic pollutant of concern with the current global plastic waste burden (Chure et al., 2021), and polystyrene (PS), a versatile, lightweight, durable, low cost and stable plastic, has assumed a significant chunk of the global plastic problem, being the third most sort after synthetic plastic (Ho et al., 2018; Urbanek et al., 2020). Used PS ends up in any environment where it stays generally inert for hundreds of years (Mohanan et al., 2020). However, styrene monomers and oligomers, as well as additives/plasticisers can leach from the PS products into the environment and compound their adverse ecological outlook (Tian et al., 2020; Wu et al., 2020).

Biodegradation of polystyrene (PS) by larvae of insects of the order Coleoptera has been confirmed in some studies and linked to the activities of intestinal microbiota, that cause chain cleavage and consequent depolymerization (Yang et al., 2014; 2015; Machona et al., 2022). Though plastics are generally regarded as inert, plastic degradation has been observed in nature, albeit slowly (Immanuel et al., 2014; Ibiene et al., 2013). The process is understood to be driven by microbial oxidation, with enzymes of the class hydrolases reported to be involved in the depolymerization process (Xu et al., 2021, Yang et 2015; Gewert et al., 2015).

The study by Yoshida et al. (2016) verified that plastic can be completely depolymerised by microorganisms. While research to bring about the complete mineralization of most plastics into carbon dioxide and water by microorganisms is gaining traction, it is becoming evident that new biomass can be produced from plastic waste with synchronous production of high-value by-products (Grima et al., 2000; Montazer et al., 2019). Among these products is
polyhydroxyalkanoate (PHA), a compound with properties which is currently being utilized for the production of biodegradable plastic.

The larvae of the African palm weevil (Rhynchophoros phoenicis) are voracious and can feed on hard palm fibre (Immanuel et al., 2022). In the present study, we exploited the degradation of PS by bacteria isolated from the gut of R. phoenicis larvae and investigated the possibility of coupling its degradation with PHA production.

Material and Methods

Sample collection.

African palm weevil larvae were collected in September 2022 from dead palm logs in the Toru-Orua community, Bayelsa State, Nigeria. Polystyrene foam was obtained from A-Z Electronics, Yenagoa, Bayelsa State. Samples were transported to the microbiology laboratory at the Department of Biological Sciences University of Africa, Toru-Orua.

Feeding Trial

A total of 100 larvae were allowed to adjust to the environment for an initial 24 hours following arrival in a plastic vessel containing the palm pith from which they were picked up. Subsequently, the larvae were split into five groups with 20 larvae each in 180 ml polyethylene containers. Group 1 was fed solely with palm pith. Group 2 was fed with 50% palm pith, 25% polystyrene and 25% brewer waste. Group 3 was fed with 80% palm pith, 10% polystyrene and 10% brewer waste. Group 4 was fed with 50% palm pith and 50% polystyrene. Group 5 was fed solely with polystyrene. The feeding trial was carried out at room temperature (28±2°C) and humidity of 80±4%. The survival rate of larvae was calculated once a week, for three weeks.

Dissection of Larva

The surviving larva after a three-week feeding trial with 100% polystyrene was disembowelled. The gut dissection was conducted in a clean Petri dish (90 mm in diameter) containing sterile normal saline with sterilized scissors and forceps. The gut was pulled out and suspended in a sterile ringer solution.

Isolation of Gut Bacteria

The gut was transferred into 10 mL of normal saline and vortex mixed for 15 minutes. One (1 mL) aliquot of suspension was transferred into a sterile test tube containing 9 mL of distilled water.

Traditional ten-fold serial dilution and agar plating method was used for the isolation of the gut microbiota using nutrient agar. A measured 0.1 mL of serial dilution was spread on the culture media using a sterile spread rod and plates were incubated at room temperature (28 ± 2°C).

Characterisation and Identification of Isolates

Biochemical characteristics of isolates were determined using API 20E strips. Isolates were

identified based on their 16S rRNA sequences.

Polystyrene film Preparation

Polystyrene foam was made into a film using the erstwhile method of Yang et al. (2015) with modification. The foam was dissolved in acetone (1mL) and then the solution was spread in a Petri dish and left for 3h to form a film. The film was removed from the Petri dish, rinsed with distilled water, and allowed to dry before use.

Polystyrene Biodegradation

A pre-inoculum was prepared in a liquid medium and allowed to grow for two weeks before being used in the biodegradation assay according to Oliveira et al (2020). The culture medium composed 0.6 K2HPO4.3H20; 0.2 KH2PO4; 0.2 MgSO4.7H2O; 0.2 (NH4)2 SO4;
supplemented with 2.0mL per litre of trace element solution containing 0.10 ZnSO4. 7H20; 0.03 MnCl2. 4H20; 0.3 H3BO3; 0.2 CoCl2.6H20; 0.01 CuCl2.2H20; 0.02 NiCl2.6H20; 0.03
Na2MO4.2H20, dissolved in 1000 mL of 0.1 M phosphate buffer of pH 7. The biodegradation assay was performed using 200 mL of the medium and 3.0g polystyrene film in a 250 mL conical flask which was shaken on a rotary shaker at 30°C and 100 RPM for 60 days.

Monitoring of Polystyrene Film Biodegradation

To monitor the PS biodegradation process, the absorbance of the assay was measured once a week at 560 nm. The biodegradation of the PS film was analysed by weight loss. Fourier Transform Infrared (FTIR) (Agilent T) spectroscopy of the polystyrene film was carried out before and after the assay to confirm surface chemical changes. The sodium hypochlorite- chloroform extraction method was used to ascertain the presence of PHA in the medium.

Results

Feeding Trial

Table 1 shows the survival rates of R. phoenicis larvae under different feed trials. The survival rate after 7 days ranged from 40% to 90%; 10%-75% after 14 days and from 5%-60% after
21 days.

Table 1: Survival rates, % (n=20)

Day	Group 1	Group 2	Group 3	Group 4	Group 5
7	90±1.5	55±2.1	75±0.9	50±1.3	40±0.2
14	75±0.9	45±1.4	60±3.6	20±0.8	10±0.1
21	60±1.8	30±2.5	45±1.6	10±0.8	5±0.2

Biochemical Characteristics and Identity of Isolates

Table 2 shows the biochemical profile of the isolates. Table 3 shows the references match and accession number of isolates.

Table 2: Biochemical characteristics of isolates

Test	Isolate A1	Isolate B1	Test	Isolate A1	Isolate B1
O-nitrophenyl-b-D-galactopyranoside (ONPG)	-VE	-VE	Gelatinase (GEL)	+VE	+VE
Arginine dihydrolase (ADH)	+VE	+VE	Glucose (GLU)	-VE	-VE
Lysine decarboxylase (LDC)	+VE	+VE	Mannose (MAN)	+VE	+VE
Ornithine decarboxylase (ODC)	+VE	-VE	Inositol (INO)	-VE	-VE
Citrate (CIT)	+VE	+VE	Sorbitol (SOL)	+VE	+VE
Hydrogen sulphide (H2S)	-VE	-VE	Rhamnose (RHA)	-VE	-VE
Urease (URE)	+VE	+VE	Sucrose (SAC)	+VE	+VE
Tryptophan deaminase (TDA)	-VE	-VE	Melibiose (MEL)	-VE	+VE
Indole (IND)	-VE	-VE	Amygoblin (AMY)	+VE	+VE
Voges-proskauer (VP)	-VE	-VE	Arabinose	-VE	-VE

Table 3: References match and accession number of isolates.

Isolate code	Similarity (%)	Reference match	Accession number
A1	95.07	Pantoea dispersa MZ562882	OQ652023
B1	99.32	Lysinibacillus macriodes KX396054	OQ652017

Biodegradation of Polystyrene

Table 4 shows that the co-culture of Pantoea dispersa and Lysinibacillus macriodes caused an 8.8% reduction in the weight of polystyrene. Table 5 shows the absorbance of the medium during polystyrene biodegradation increased from 0.142 (day 1) to 0.542 on day 60. Figures 1 and 2 show FTIR spectra of polystyrene film before and after biodegradation respectively.

The isolates were able to produce PHA equivalent to 1.4g/L, under PS degradation conditions.

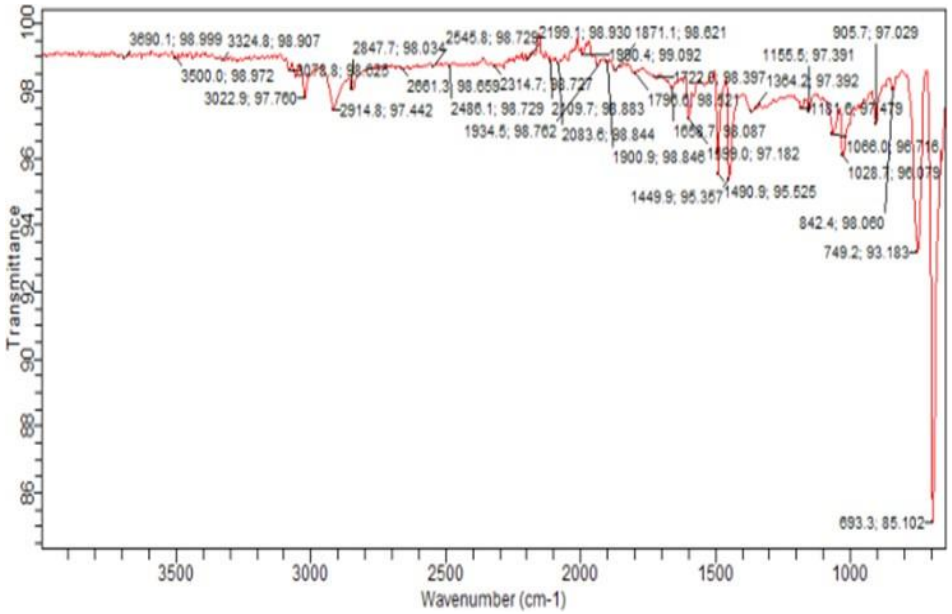

Fig.1: FTIR spectra polystyrene film before biodegradation

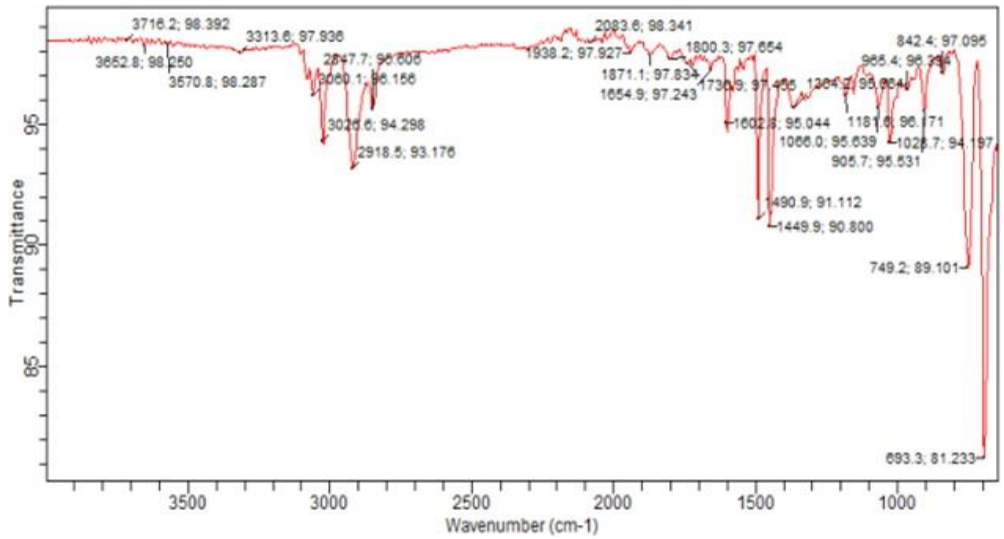

Fig. 2: FTIR spectra polystyrene film after biodegradation

Table 4: Weight loss of polystyrene film

Inoculum	Initial weight of PS (g)	Weight of PS after 60 days (g)	% weight loss
Pantoeadispersa + Lysinibacillus macriodes	3.0	2.736	8.8

Table 5: Absorbance readings during polystyrene biodegradation assay

Time (Day)	Absorbance (540nm)
1	0.142
7	0.208
14	0.228
21	0.263
28	0.284
35	0.304
42	0.356
49	0.443
56	0.523
60	0542

Discussion

In line with the search for solutions to plastic pollution, the production of biodegradable bioplastic and the development of innovative processes for the valorization of plastic, are considered the most appealing approaches (Oliveira et al., 2020). Thus, this work investigated the biodegradation of PS in the gut of R. phoenicis, examined by in vitro degradation of PS by co-culture of bacteria isolated from the gut of weevil larvae that survived solely on the PS diet.

From the feeding trail, it was observed that the larvae had the highest survival rate (90%, 75% and 60%) when fed with their natural diet of palm pith and the lowest survival rate (40%, 10% and 5%) when fed with only PS. Expectedly, the survival rate

of the larvae was highest in palm pith (their natural diet) and lowest in 100% PS. Nevertheless, it was reasoned that the continued existence of the larvae solely on the PS diet could be a thinkable suggestion that the polymer was biodegraded, and this could have been assisted by the microorganisms present in the gut of the larvae. This is in line with the argument by Yang et al. (2018) that faecal extracts of yellow worm larvae showed alteration of excreted PS, evidenced by changes in carbonyl groups and hydroxyl groups of the polymer, indicative of biodegradation.

A total of eight (8) bacteria were isolated from the gut of R. phoenicis larvae after dissection and cultivation. Among them, only two isolates were able to grow on a minimal medium containing PS as the only carbon source. The total number of isolates is much lower than the previously reported number of isolates by Immanuel et al. (2022), after feeding with palm pith. This aligns with a report indicating that microbial miscellany is positively correlated to the diet of insects (Priya et al., 2012; Krams et al., 2017).

The two isolates were identified as Lysinibacillus macroides and Pantoea dispers. Jeon et al. (2021) isolated PE and PP biodegrading species of Lysinibacillus sp. from a soil grove. Pantoea dispersa for the degradation of plasticizer which they attributed to enzymes capable of ester bond hydrolysis (Xu et al., 2021). Genes for styrene degradation such as aliphatic amidase and nitrilotriacetate monooxygenase are present in some species (Olson et al., 2022)

The biodegradation of PS by the microbial isolates was confirmed by comparing FTIR spectra of PS before and after the biodegradation assay. Oxidation of a polymer group is expected after a chain splitting/degradation, which leads to the production of a carbonyl molecule, phenolic, and alkenes, as degradation products (Ibiene et al., 2013). Oxygen incorporation in carbonyl groups in esters and ketones is a typical indication of oxidative degradation of plastic (Hadad et al., 2005; Gilan et al., 2004). The presence of carbonyl groups shows up as absorption peaks in the range of 1640-1760 cm-1. The absorption peak height indicates the degree of oxidative degradation in the plastic (Immanuel et al., 2014). The -OH stretching region (3000-3700 cm-1) incremented in the PS film exposed to the bacterial consortium. Ibiene et al. (2013); and Guadagno et al. (2001) in their work on

polyethene degradation also observed an increase in the stretching region of a hydroxylic group, which they attributed to the formation of degradation products such as hydroxyperoxide and alcohol. Increased signal in the carbonyl groups after microbial treatment of PS in association with removal -OH bounded compounds, were deduced as evidence to support degradation by weight loss as the peaks were greater for treatments which gave higher loss in weight since alcohol, hydroxyperoxide, ketones, esters and carboxylic acids and any other low molecular weight degradation products are easily assimilated by microorganisms (Hakkarainen and Albertsson, 2004).

Although a chemical change in the PS film incubated with the bacteria isolates is used to confirm PS film biodegradation, the most express suggestion of PS degradation is weight loss (Yang et al., 2015; Kim et al., 2020; Wang et al., 2020). Co-culture of P. dispersa and L. macriodes caused an 8.8% loss in weight of PS film. The fraction degraded is low compared to the fraction left unaltered. This is because microbial degradation of plastics is affected by their high chemical stability and low bioavailability (Yang et al., 2014). However, the amount of plastic degraded is much higher than previous report by Jiang et al. (2021) having a weight loss of 1.05% after incubation with Massila spp. For 30 days. Likewise, Wang et al. (2020) reported a total weight loss of 1.4% after 60 days of cultivation with Acinetobacter spp. AnTC-1. In addition, Mor and Sivan (2008) and Yang et al. (2018) reported a weight loss of 0.8% in 56 days of incubation with Rhodococcus C208. But lower than values reported by Mor and Sivan (2008) and Yang et al. (2018) having a weight loss of 7.4% after cultivation with Exiguobacterium sp. YT2 for 60 days.

During the biodegradation assay, the absorbance of the medium varied from 0.142 (Day 1) to 0.542 (Day 60). In addition, a turbid liquid was detected in the medium, which indicates that PS biodegradation might have occurred and that isolates were able to utilize PS as their sole carbon source, as suggested by Jiang et al. (2021). However, the optical density of the medium during the biodegradation period could suggest microbial utilization of styrene monomers and/or hydrolysable plastic additives (Xu et al., 2021).

The isolates were able to accumulate PHA equivalent to 0.2g/L. This is lesser than yield of 0.9-1.1 g/L reported by Dienye et al. (2022) by strains of Bacillus velezensis, Bacillus paramycoides, Lysinibacillus capsici, Lysinibacillus macriodes and Priestia flexa isolated from the same Niger Delta region as the present study, although under optimized condition, using easily hydrolysable agro-waste. Coupling biodegradation of plastics with the production of PHA by bacteria was reported by Oliveira et al. (2020) as a mitigation strategy for plastic waste as well as valorization.

Conclusion

This study isolated and identified PS-degrading strains of Lysinibacillus macriodes and Pantoea

dispersa, from the gut of African palm weevil (Rhynchophorus phoenicis) larvae. The isolates were able to degrade PS and accumulate PHA in their cells. The study can be optimized for the recycling and valorization of plastics.

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