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Polystyrene biodegradation by Pseudomonas sp. strain PVGSPCDU isolated from Superworm gut

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ABSTRACT

Massive amounts of plastic garbage are a serious social problem. There have been several initiatives to address the issue of plastic trash, including techniques for the natural decomposition of plastic. Since polystyrene (PS) is currently one of the plastics most often utilized in numerous industries, its degradation is a serious worldwide concern. In this study, we identified a Pseudomonas sp. Strain PVGSPCDU bacterium that breaks down PS from the superworms' guts. The breakdown of PS by Pseudomonas sp. has hardly been investigated up to this point. Using electronic microscopy, we investigated PS degradation and recorded atomic distribution and contact angle changes with water droplets on the PS surface, which indicate a chemical transition from hydrophobicity to hydrophilicity. Using Fourier transform-infrared spectroscopy (FT-IR), we investigated chemical structural changes during PS degradation by Pseudomonas sp. to look for the formation of C=O bonds and shifts towards hydrophilicity. According to our research, Pseudomonas, which is found in the superworms' guts, contributes to the breakdown of plastics after consumption. Therefore, the results of this study are important because they show a possible remedy for PS degradation in addition to revealing a new role for Pseudomonas in superworms' guts..

Keywords: polystyrene, plastic, degradation, superworm, Pseudomonas sp..

1. INTRODUCTION

Plastic is becoming one of the most extensively utilized materials due to its sharp rise in use worldwide. But because natural plastic decomposition is so slow, plastic garbage builds up and becomes a serious social problem. Plastic treatment consists of 77% reclamation, 13% incineration, and 10% recycling due to the absence of a degrading technique (Kim et al., 2020).

A typical petroleum-based thermoplastic with a high molecular weight and a very stable structure is called polystyrene (PS).In 2019, PS's global manufacturing capacity was 15.61 million metric tons, but about 370 million tons of plastic were manufactured worldwide during the same year (Plastics Europe).Since PS is one of the most widely used polymers for food packaging, it is utilized in a wide range of industries and daily life.PS is available in two forms: foam and stiff. While the foam form (styrofoam) is frequently utilized to create single-use containers, like throwaway food boxes or cups, the rigid form is used in clear food containers (Ho et al., 2018).PS containers are misused because they are inexpensive and accessible.PS endures for extended periods of time as solid waste because it is a robust thermoplastic that takes several hundred years for natural ecosystems to completely degrade (Bandyopadhyay and Basak, 2007).

The biodegradation of plastic by microorganisms has gained attention recently as a viable green method for getting rid of plastic trash. The bulk of the microorganisms that were examined for their ability to degrade plastic were isolated from soils, oceans, and landfills (Ho et al., 2018). Fungi and bacteria accounted for the majority of PS-degrading microorganisms (Chaudhary and Vijayakumar, 2020; Ho et al., 2018; Yanto et al., 2019).

The predominant bacterial genera Pseudomonas and Bacillus may break down a variety of plastics, including PS (Matjašič et al., 2021).

Recent research, however, found that a variety of insect larvae might consume plastic and aid in its breakdown. Notably, it was reported that mealworms (larvae of Tenebrio molitor L.) (Yang et al., 2015a, 2015b; Brandon et al., 2018; Brandon et al., 2021), superworms (larvae of Zophobas atratus L.) (Peng et al., 2020; Yang et al., 2020), Greater Wax Moth larvae

(Galleria mellonella L.) (Lou et al., 2020), and Plesiophthalmus davidis larvae (Woo et al., 2020) could consume PS.

The intestinal bacteria and the potential for plastic degradation of the mealworms and superworms were decreased when they were fed antibiotic-containing bran. These suggested that the gut microbiota played a role in the breakdown of plastic (Yang et al., 2015a, 2015b). Numerous bacterial species were isolated from worm guts and identified as plastic-biodegrading strains, including Serratia sp. strain WSW from Plesiophthalmus davidis (Woo et al., 2020), Pseudomonas sp. strain DSM 50071 from superworms (Kim et al., 2020), and Exiguobacterium sp. strain YT2 from mealworms (Yang et al., 2015a, 2015b).

The current study looks for PS-degrading bacteria in Z. atratus larvae (the superworms' guts).Three bacterial strains were chosen for investigation based on their capacity to degrade PS on their own.PS degradation was assessed by looking at alterations in surface morphology (SEM) and chemical modification (FTIR)..

2. MATERIALS AND METHODS

Preparation of Styrofoam and PS film materials

The Styrofoam (expanded polystyrene foam) feedstock was prepared by breaking down the commercial Styrofoam sheets into smaller pieces. No additives were added to the Styrofoam.

The PS film was prepared by dissolving 0.15 g of Styrofoam in 5 ml of xylene. The dissolved Styrofoam was poured into a glass dish (9 cm diameter plate) and dried in a fume hood for 3 days. The films were removed, rinsed with ethanol and distilled water and dried in a sterile

hood. The dried films were kept in an aluminium foil-wrapped desiccator prior to usage.

Feeding and Extraction of gut

Superworms, the larvae of *Zophobas auratus*, were purchased from Pisces, Kolkata, India.Moisture was controlled with a custom water sprayer to create a fine mist layer on the underside of the superworm container lid. All experiments were carried out at room temperature, which ranged from 20 to 25 °C during the experiment. After the acclimatization period, the larvae were only fed with Styrofoam for a period of 1 month. For gut bacteria isolation, 30 larvae (0.4 g per each on average) fed with Styrofoam as a sole diet for 30 days were anaesthetized by immersing in 75% ethanol for 1 min and then rinsed twice with sterile saline water (0.85% NaCl solution). Their guts were drawn out and placed in a 15-ml tube containing 3 ml of sterile saline water. The tubes were then vortexed for 5 min, and the gut tissue was removed. The gut suspension was used for the screeningof PS-degrading bacteria by transferring to a 250 ml Erlenmeyer flask that contained 1 g of small Styrofoam pieces and 80 ml of Liquid Carbon Free Basel Medium containing (gL^{-1}): (0.7) KH_2PO_4, (0.7) K_2HPO_4, (0.7) $MgSO_4 \cdot 7H_2O$, (1.0) NH_4NO_3, (0.005) NaCl, (0.002) $FeSO_4 \cdot 7H_2O$, (0.002) $ZnSO_4 \cdot 7H_2O$, (0.001) $MnSO_4 \cdot H_2O$.The flask was incubated on a rotary shaker (120 rpm) at ambient temperature for 60 days. One hundred microlitres of the gut flora mixture was spread across plate with Luria–Bertani

agar medium and incubated at 37℃. After culturing for 24 h, colonies obtained were picked and steaked on fresh LB agar medium until single colonies were obtained. Each type of bacterial single colony was classified by their morphology and used for the screening of the PS-degrading ability.

Preliminary screening of isolates for the PS-degrading ability

Each type of colony was inoculated in a 5 ml LB liquid medium and incubated at 37°C for 16h. Then, the culture was centrifuged at 8000 *g* for 5 min to obtain the cell pellet. The pellet was rinsed with LCFBM three times to ensure that no LB medium was left in the bacterial cells. Then, the cell suspension (0.5 ml) with a concentration of approximately 10^8 cells per ml was inoculated into LCFBM (50 ml) supplement with the PS film (3.0 × 3.0 cm) as acarbon source. LCFBM without the PS film and LCFBM without inoculated bacterial cells were used as controls to determine whether the cells could grow on LCFBM alone. All culture flasks were incubated at 30°C on a rotary shaker (120 rpm) for 30 days. The biofilms formed on the PS film sheets were used as a characteristic to screen for potential PS-biodegrading strains. After the incubation time, bacterial-treated films were collected from the liquid culture broth and boiled in 5 ml 0.5 mol−1 NaOH for 30 min. The suspension was centrifuged and the pellet and the supernatant were separated. The protein content in the supernatant was quantified through the Lowry assay to estimate the amount of bacterial colonies that were able to form a biofilm on the PS surface (Lowry *et al.,* 1951).

PS film biodegradation

Each selected strain was grown on LB medium for 16 h. The culture was centrifuged to collect the bacterial cells, and the supernatant was discarded and replaced with 5 mlof LCFBM, which was vortexed to homogenize the solution. After repeating the centrifugation step three times, the cell suspension with a concentration of approximately108 cells per ml was spread on a solid CFBAM plate and three pieces of PS film cut in uniform size (1.50 × 1.50 cm) were placed on top of the bacterial cells. The negative control was prepared by excluding the bacterial suspension from the setup. The experimental and negative-control were then incubated for 30 days at 30°C.

Maintenance of cultures

All isolates having the capability to degrade polystyrene in the above methods were maintained on Luria Bertani agar slopes as working cultures. Culture stocks were also maintained on Luria Bertani agar slopes, by sealing the tubes with paraffin wax. Preservation of cultures at 4°C was achieved by growing the isolates in 0.5 ml half strength Luria Bertani broth in sterile capped vials. Glycerol was sterilized and 0.5 ml was added to the grown culture as a cryoprotectant and the vials were preserved at 4°C.

Identification of the selected isolate

The selected isolate was classified using routine biochemical methods/ techniques as per the Bergey's manual of determinative bacteriology (Krieg and Holt, 1989; Sneath *et al.,* 1989).Following the extraction of the bacterial genomic DNA, genomic PCR was carried out to identify the species of bacteria cultivated in the liquid medium. To identify the species, 16S rRNA gene sequence analysis was performed using the following primers: Reverse primer 1492-R (5′-GGTTACCTTGTTACGACTT-3′); forward primer 27-F (5'-AGAGTTTGATYMTGGCTCAG-3'). Following 16S rRNA DNA sequencing, NCBI BLAST analysis was used to identify the type of bacterium.

Analysis using Scanning Electron Microscopy (SEM):

PS breakdown and the bacterial proliferation on the PS surface were confirmed using a SEM (SU8230, Hitachi, Japan). The PS that bacterium had grown on was cut to a size of 1 cm × 1 cm, and the surface was examined. The bacteria adhered to PS were removed using a 2% sodium dodecyl sulfate (SDS) solution for four hours in order to examine shape change during PS breakdown. The PS surface was scanned at 1.6 kV acceleration voltage in order to detect deterioration and structural changes.

Examination of PS using Fourier-Transform Infrared Spectroscopy (FT-IR):

Thermo Fisher Scientific's FT-IR was utilized to ascertain the modifications to the chemical structure of the biodegraded PP brought on by the bacterial species. PP biofilm was cleaned twice with deionized sterile water and 2% SDS to get rid of microorganisms. Using a diamond tip with a range of 3500–500 cm^{1}, the clever single-bounce attenuated total reflectance was employed.

Quantification of biodegradable plastic bead mass reduction:

One gram of PS plastic bead was used to compare the bacteria-mediated biodegradation efficiencies, calculated in the form of mass decrease.For 15 days, biodegradation reactions were carried out in liquid LCFBM at 35℃ while shakingat 180 rpm.Biodegradable plastic beads were gathered using a 0.45 m filter and a 70 m sieve. After soaking in 2% SDS for three times, the germs that had adhered to the beads were eliminated by DW rinsing. Mass reductions of various plastic bead types were measured separately in triplicate after being dried for 24 hours at 60 ℃. The liquid LCFBM with plastic bead but without bacteria was tested as a control under the identical circumstances.

Evaluation of bacterial growth using colony counting and optical density (OD) Measurement:

Bacteria were inoculated in 25 mL of liquid LCFBM media containing 2 g of PS plastic bead in order to examine the bacteria-mediated biodegradation efficiencies. To assess the bacterial growth rate daily for eight days, the optical density (OD) of 1 mL of liquid LCFBM media containing bacterial growthwas measured at 600 nm. Additionally, a colony census was conducted. One mL of liquid LCFBM medium containing bacteria and PS plastic beads was serially diluted, then the liquid medium was spread out onto a nutrient solid media plate. The number of colonies that grew was then counted after successful growth.

3. RESULTS AND DISCUSSIONS

The polystyrene (PS) biodegradation is known to progress very slowly in natural ecosystems,and requires a very long time for completion of its degradation. There are many reports on insects which are able to digest various plastics. For instance, mealworms were first reported to ingest and mediate rapid PS Styrofoam degradation. Suchinsect-based systems suggest that PS degradation can occur within a relatively short time. Inaddition to the well-known mealworms, superworms were also characterized to mediate PS degradation via ingestion.

Survival, behavior, and life cycle of the host

Superworms from three groups-vegetable fed, PS-reared, and a starving group-participated in a three-week feeding trial after an initial one-week acclimation period. Throughout the feeding study, all three groups' superworm survival rates were about70% (Fig.1)after three weeks. This could be explainedbecause of the superworm densities that were more than ten times greater (Yang *et al.,* 2020). By preventing overcrowding, which has been linked to aggressive behavior and worm deaths, as shown for the common mealworm (Weaver and McFarlane, 1990), the survival rate to be drastically improved to nearly 98%.

Fig. 1 The three groups after 3 weeks of respective feeding.

During the first 24 hours of the experiment, the PS group's superworms easily approached the PS blocks and chewed their way inside to form narrow burrows (Fig. 1A, B). We made several anecdotal findings but did not measure superworm activity. Throughout the experiment, worms on the PS diet stayed active, albeit more slowly than the worms in the vegetable fed group. With extended periods of rest punctuated by brief investigations, the starving group that was not fed moved the least. During the first 24 to 48 hours in the PS group, the worms' feces changed from light brown to white pellets, indicating that they had begun to ingest and digest PS. Throughout the experiment, the average weight of the superworms in the bran group more than doubled (0.3 g per worm to 0.7 g per worm), resulting in noticeably heavier worms ($P<0.001$) than those in the starvation and PS groups. Throughout the feeding session, the PS group's average superworm weight (0.3 g per worm to 0.45 g per worm) marginally roseleading to a slight but noteworthy increase in weight ($P<0.001$) at the conclusion of the feeding session. The anecdotal evidence of increased activity in comparison to the starvation group and the weight gain indicate that the worms in the PS group were able to get energy from the PS diet, most likely in collaboration with their microbial gut populations. Prior reports of carbon recovery efficiency, which showed that the superworm gut is where ingested PS mineralization takes place, lend credence to this theory (Yang *et al.,* 2020).We then examined the superworms' microbial gut ecology to find microorganisms, encoding pathways, and enzymes that might be involved in the breakdown of PS.

Fig. 2. PS fed group formed burrows after 24H of initiation of the experiment (A), chewed almost 90% of the PS board after 30 days (B).

To compare the changes in theingested amount of PS supplied, we examined changes

in the weight of PS, which was almost same as that of the group that is fed on the normal diet (green vegetable leftovers and bran etc.). During a period of one month, the PS block was almost chewed upto 90% (Fig. 2B) by the superworm group which was exclusively fed by PS Styrofoam. Maintenance of sufficient humid conditions by way of 2-3 water sprays daily and cool dark conditions increased the amount of PS ingested by this superworm group. The age of the superworm larvae also play a role in PS ingestion, since old larvae rapidly transform into adult pupa, they do not show such capability of ingestion. Another factor involves in the amount of ingestion was found to be the number of larvae in each of the closed system (or box we used in the study). Overcrowding leads to cannibalism which is a normal phenomenon in most of the insect larvae.

Bacteria that degrade PS have been isolated, identified and reported in numerousprevious studies, where most PS-degrading microorganisms have been initially reported to exist in the soil. However, there are numerous recent studies that have reported that the gut bacteria in insect larvae like superworms are directly involved in the plastic degradation. This has been confirmed using various methods including enrichment in presence of antibiotic compounds which yielded no bacteria thus establishing the role of gut microbes in plastic degradation beyond any doubts (Yang et al., 2020).

As superworms readily ingested the PS in our experiment, we anticipated that they may also contain active PS degradation bacteria in the gut, as established in a lot of previous reports. To confirm this, the guts of superworms were extracted surgically, andbacteria inhabiting the gut were isolated for identification. Because a huge diversity of bacteriasurvives in the guts of the superworms, it was deemed likely that some, but not all would bedirectly involved in PS degradation. We also performed a metagenome 16S rDNA enriched Illumina sequencing for the gut samples (data not shown here). To identify the specific bacteria directly degrading PS, thesuspension was first cultured in PS-containing LCFBM liquid medium for two months after gutextraction. After spreading 2 mL bacteria containing LCFBM solution on the solid nutrientmedium and culturing them at 28-30 °C for 7-10 days, we found five different strains of bacteriagrowing on the solid medium, each showing distinct morphology.

Bacterial colonies started to appear on the surfaces of PS films after 7-10days. A few randomly selected colonies based on their rapid growth in comparision to others and distinct colony morphological features were subjected to further studies for their identification, which include both biochemical characterization and molecular identification using 16S rDNA sequencing. Among these five isolates (other strain data not shown here), one major type of bacteria which grew in a large-sized oval form with a translucent was much larger than that of the other bacteria. After 10 days, this bacterium was enriched further which led to further increase in the growth. Based on 16S rRNA sequencing analysis, the bacterial cells of this specific colony were identified as *Pseudomonas* sp.(Gen Bank: PV849392.1). The phylogenetic analysis was performed by distance-based technique, neighbor-joining (NJ).The Kimura correction was used to calculate distances in a pairwise deletion fashion (Kimura, 1980). Using the MEGA6 program (Tamura *et al.,* 2013), neighbor-joining (NJ), technique was employed to create phylogenetic trees. A bootstrap process was used to obtain the percentage support numbers (Fig. 3). Therefore, *Pseudomonas* sp. both survived and thrived under a nutrient-deficient environment with PS as the sole carbon source.

In the incubation with PS beads in LCFBM solution, numerous *Pseudomonas* sp. were attached on the surfaces of the PS beads and the amplification of bacterial populations via cell division was also observed. After removing most attached bacteria with SDS treatment, PS beads still had a few remaining bacteria adhering, and showed a strong surface corrosion after biodegradation in liquid LCFBM media for 20 days, unlike the control group without bacteria.

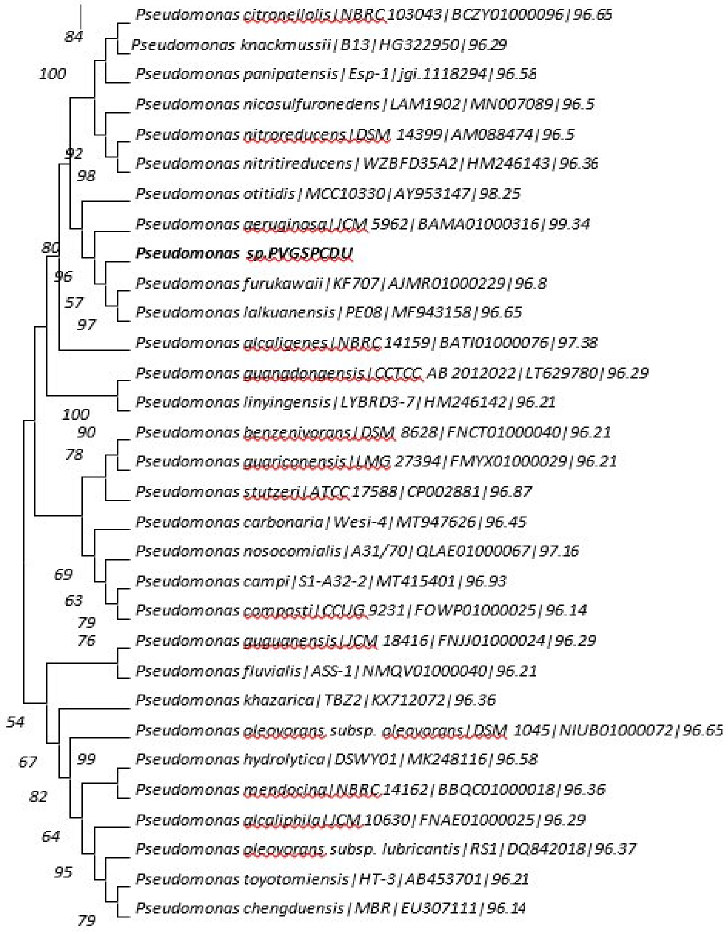

Fig. 3.Phylogenetic neighbour joining tree of strain *Pseudomonas* sp.PVGSPCDU

FT-IR Analyses of PS Biodegradation by Pseudomonas sp.

The weight reduction measurements showed that the *Pseudomonas* sp.-mediated biodegradation of PSonly proceeded very slowly. To check the biodegradation of PS by Pseudomonas sp. , we further conducted FT-IR to analyze the chemical structural changes of PS beads. Compared to PS in the control, representative absorptions of carbonyl (−C=O) at 1715 cm^{-1} and hydroxyl (−O–H) at 3300–3600 cm^{-1} identified that Pseudomonas sp. -mediated PS biodegradation had occurred (Fig. 4).

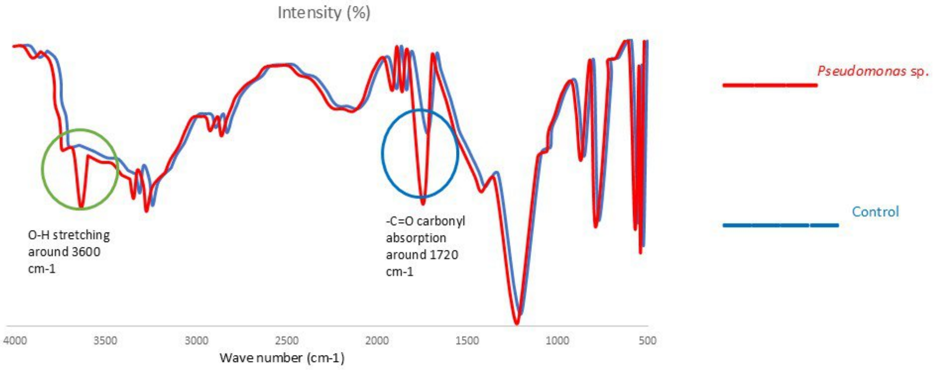

Fig. 4.FTIR structural analysis of PS degraded by *Pseudomonas* sp.PVGSPCDU

SEM Analyses of PS Biodegradation by Pseudomonas sp. PVGSPCDU

SEM was used to examine the shape and characteristics of growing colonies, and the obtained images showed that *Pseudomonas* sp. firmly attached to the PS surface (Fig. 5). The viability and proliferation of *Pseudomonas* sp. on the PS surface indicated that PS degradation could be utilized as the energy resource in the absence of other alternate carbon resources. After 60 days of culture on PS-LCFBM solid medium, *Pseudomonas* sp.attached to the surfacewas removed with SDS containing buffer, and the PS surface was examined using SEM. The edges of PS were changed to a smooth form as a result of degradation by *Pseudomonas* sp.whereas, the edges remained rough in the control. In addition to edge smoothing,holes formed by PS degradation were observed on the PS surface where colonies had beenpresent, further confirming PS degradation by *Pseudomonas* sp.Despite of SDStreatment, a proportion of *Pseudomonas* sp. were not removed completely in or near such holes. Our findings provide the direct evidence that *Pseudomonas* . isolated from the guts of superworms degrades PS.

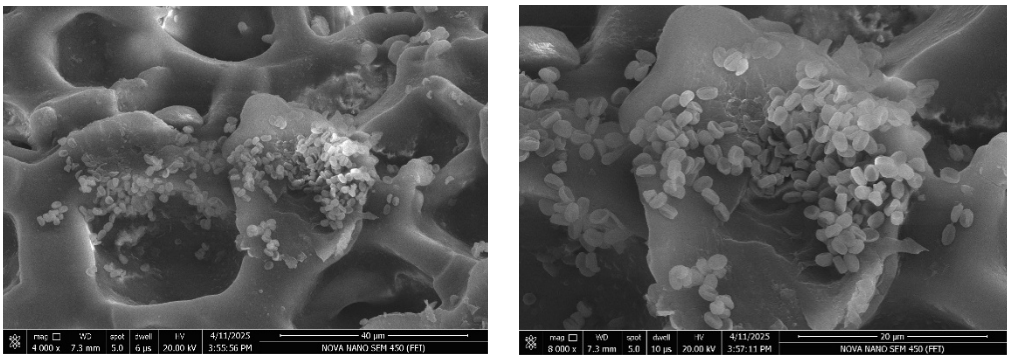

Fig. 5.SEM analysis of PS degradation by *Pseudomonas* sp. PVGSPCDU.

Many bacterial species reside in insect larvae guts and participate in the biodegradation of ingested plastics (Kim et al., 2020; Urbaneck et al., 2020). However, due to inevitable environmental differences, including oxygen level, pH, and ion concentrations between the gut environment of the insect larva and our in vitro enrichment systems, the most efficient plastic-biodegrading bacteria in the gut are not always successfully isolated or match bacteria that thrive in our in vitro enrichment methods. For instance, the most effective PS-biodegrading anaerobic bacteria strains in the gut of insect larva are lost when their gut transits into an in vitro environment provided with oxygen. However, P. aeruginosa are classified as aerobes or facultative anaerobes and able to survive and grow well in both aerobic and anaerobic environments (Wu et al., 2005; Alvarez-Ortega and Harwood, 2007), including soil, wood, water, dumpsites, the deep sea, and guts of larvae of superworms which suggests *Pseudomonas* species are highly adaptive. They not only degraded plastic in the guts of larva but also in our in vitro systems with plastic-added LCFBM media.

In this study, we demonstrated that *Pseudomonas* sp. is capable of biodegrading PS. The *Pseudomonas* sp. strain used was obtained by isolating gut extracts from superworms that had consumed PS, and then culturing the extract in PS-supplemented liquid LCFBM medium for 60 days. Both our current findings and previous research indicate that a single bacterial strain can break down multiple types of plastic, though the efficiency varies among them (Li et al., 2020).

Plastic biodegradation efficiency largely depends on the quantity of enzymes produced and the catalytic effectiveness of those secreted enzymes (Yoshida et al., 2016; Austin et al., 2018; Palm et al., 2019). Through hydrolysis, these enzymes can alter the plastic surface from hydrophobic to hydrophilic, which weakens its mechanical strength (Santo et al., 2013). Recent research has identified several enzymes involved in plastic degradation, including serine hydrolase (SH) secreted by many *Pseudomonas* strains. There has been a lot of reports which have also emphasized the importance of oxidation as a key initial step in bacteria-driven plastic degradation, promoting subsequent stages such as biodeterioration, biofragmentation, assimilation, and mineralization (Singh and Sharma,2008; Shimao,2001).

When plastic is provided as the only carbon source, bacteria must derive energy from the byproducts of plastic degradation to support cellular functions, build cellular components, and enable cell division for reproduction (Wierckx et al., 2015; Bode et al., 2000). The breakdown products of PS can be readily converted into fatty acids through enzyme-catalyzed oxidation processes, which are then further processed into acetyl-CoA for energy production via β-oxidation and the TCA cycle (Mooney et al. 2006). Breaking PS ring to form intermediates like acetyl-CoA or succinate for entry into the TCA cycle, requires more enzyme-driven reaction steps (Ru et al., 2020). The complexity of these enzymatic processes may contribute to the slower degradation rates of PS. This could be the reason for the less efficient breakdown of PS which resulted in limited energy production, thereby slowing the growth of *Pseudomonas* sp. Interestingly, the bacterial population was very high in the presence of PS (Fig. 5), even though PS degraded more slowly. Several earlier findings highlight how the distinct chemical structures of different plastics such as, PE, PS, PPS, and PP necessitate different enzymatic pathways for degradation by *Pseudomonas* species, which in turn results in varied biodegradation efficiencies. While faster degradation theoretically yields more carbon fragments for building cellular structures and generating energy, the effectiveness with which these fragments are converted into usable biological products also plays a critical role in supporting bacterial proliferation (Mihreteab et al., 2019).

4. CONCLUSIONS

This study revealed the remarkable ability of *Pseudomonas* sp. isolated from superworm guts, to biodegrade PS plastic. However, the degradation efficiency may differ depending on various factors. We propose that both the biodegradation speed and the efficiency with which degradation by products are transformed into energy and cellular building blocks collectively influence bacterial growth. The selective degradation patterns exhibited by a single PS bacterial strain could potentially be applied in the final purification steps of plastic recycling processes in the future

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