PROBABLE PLASTIC BIODEGRADATION BY GUT-MICROFLORA OF WEEVIL

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Abstract: Low-density polyethylene (LDPE) plastic, although has wide industrial application, is resistant to dilute acids, alcohols, aldehydes, and ketones. It is thus a leading cause of pollution and causes an imbalance in the ecosystem. Some species of insects and weevils, by virtue of their unique gut-microbiota, are known to degrade complex polymers including LDPE. Recent studies have also shed light on the ability of 'yet uncultured bacteria' in biodegradation of complex polymers. Present study aimed at isolation of probable novel bacteria from the gut of *Sitophilus* weevil, able to degrade LDPE, using special cultivation media.

Keywords: LDPE, Insect gut-microbiota, *Sitophilus spp*., dilute nutrient media

INTRODUCTION

Low-density polyethylene plastic is a synthetic polymer made up of repeating units of ethene. It is a ubiquitous polymer, popular for its wide use, ease of synthesis, and consumer satisfying properties. It is used in almost all industries such as packaging and the production of common household commodities (utensils, bags, etc). Almost 6.3 billion tons of plastics were produced worldwide in 2015 and the number is increasing exponentially each year because of its efficient and versatile use (Yuan *et al*., 2020). In spite of versatile applications, plastic and related polymers have created havoc in the last few decades, being recalcitrant to chemical degradation processes. Plastic accumulation leads to flooding by choking the water draining system. It also gets incorporated into the food chain of animals and damages their digestive system. Long term plastic accumulation in soil even severely affects its microbial community structure (Gupta and Nair, 2020). Thus, the remediation of plastic is the need of the hour.

Various physicochemical methods are available to degrade plastic. Majority of them, however, lead to harmful by-products and pose threat to the environment. One of the promising sustainable solutions is biodegradation. Recent studies have implicated the role of plastic-eating worms that digest and convert plastic into non-hazardous compounds (Bombelli *et al*., 2017). Mandibulate insects have been shown to 'chew' as well as 'degrade' plastic packages, including those made of polyethylene (PE) (Riudavets *et.al*, 2007). Insect gut-microbiota and related enzymes are presumed to play a vital role in the

degradation process. Interestingly, gut microbiota of insects seems to have been evolving according to the prevalence of recalcitrant xenobiotics like plastics (Seonghan *et.al,* 2020).

Abdulhay (2020) used confused flour to show that beetle *Tribolium confusum* larvae could consume and biodegrade different types of plastics. Elijah et al. (2015) have studied and compared biodeterioration of nylon by testing three species: *Tribolium*, *Sitophilus*, and *Oryzaephilus* using three types of plastic for 6weeks. Results showed that the number of holes (as an index of biodeterioration) in the film increased with time, among which *Sitophilus* created 23-79 holes in a thin black plastic bag of 0.1mm thickness.

Sitophilus spp. are common pests in stored grain and thus are easily available. Studies have indicated the role of gut flora of *Sitophilus* weevils in degradation of cellulose and (Prasad *et al*., 2016; Prasad *et al.,* 2019). *Sitophilus* weevils have also been shown to make pores in plastic. However, to the best of our knowledge, studies on the gut-microbiota of *Sitophilus* in plastic biodegradation are lacking. Present study aimed at isolation of probable novel bacteria from the gut of *Sitophilus* weevil, involved in LDPE biodegradation, using dilute nutrient media.

MATERIALS AND METHODS

Collection of *Sitophilus* weevils

Sitophilus weevils were collected from infested wheat grain. Wheat grains were procured from a local market, Thane (Maharashtra, India). Identification of *Sitophilus* weevils was done based on morphological characteristics such as length, colour, and presence of snout and antenna.

Extraction of *Sitophilus* gut

Surface sterilization of the weevils was done using sterilized filter paper dipped in 70% alcohol. The surface-sterilized weevils were transferred to a sterile 20ml dilution tube containing sterile 1ml distilled water. The weevil gut was pulverized using a sterile glass rod to release the gut flora in water followed by homogenization by vortexing for 20-30 seconds. The above step was followed thrice to get a total volume of 3ml. After the debris had settled down, 0.05ml of the gut extract was transferred in a sterile 20ml dilution tube and used as an inoculum for culturing microorganisms.

Preparation of dilute nutrient broth

Preparation of 1:100 dilute nutrient broth was done by adding 0.013gm of nutrient broth powder (HIMEDIA) in 100ml distilled water, the prepared dilute nutrient broth was autoclaved at 121°C, 15Ibs pressure for15min.

Preparation of Test and control systems

Test system:

4.95ml of sterile dilute nutrient broth was transferred into the tube containing 0.05ml inoculum (gut extract). LDPE plastic was cut into 0.5cm × 0.5cm square pieces, surface sterilized with 70% alcohol and allowed to dry. Four surface-sterilized plastic pieces were transferred into the tube containing dilute nutrient broth and gut extract as inoculum.

Control system:

Four surface-sterilized plastic pieces were transferred into tube containing sterile dilute nutrient broth and sterile distilled water (instead of gut extract as inoculum).

Both, the test and control systems, were incubated at 37°C for 58 days.

Microscopic analysis

A loopful of suspension, each, from test and control systems, after incubation for 58 days, was used to make smear on a grease-free slide. It was further subjected to Gram staining (Colco, 2005) and cell wall staining (Webb, 1954) and observed at 1000x magnification. LDPE piece each from the test and control systems was observed under 1000x magnification as a wet mount prepared with 5% safranin.

RESULTS AND DISCUSSION

Microscopic analysis

Visual observation of the test and control systems showed presence of turbidity, indicating microbial growth, in the test system, which was absent in the control system.

In order to study the type of bacteria present in the test system, Gram staining was performed. Surprisingly, however, no microorganisms could be observed.

Further investigation involved negative staining using 1%, 2%, 3%, 4%, and 5% nigrosine dye. Microbial cells present on darkly stained background could be observed only in negatively stained slide with 5% nigrosine. Microscopic observation revealed the presence of a diverse group of microorganisms, viz. bacilli, streptobacilli, cocci, diplococci, and filamentous microorganisms.

These results were confirmed by cell wall staining of the test sample using Chance's method. The method appears to be effective on most bacteria, even on forms considered difficult to be stained, such as *Nocardia* and *Streptomyces* (Webb, 1954). Cell wall

staining of test sample revealed the presence of bacilli, cocci and diplococci-shaped microorganisms.

Sample from control system didn't show presence of bacteria in any of the staining methods.

Since negative staining and cell wall staining procedures employ no heat fixation step along with use of dyes with negative charge; we speculate presence of novel bacteria with unique cell wall composition as inhabitants of the gut of *Sitophilus* weevil.

Examination of LDPE piece in test tube under a microscope

On the 11th day of incubation, a biofilm was observed in the test system adhered at the bottom of the test tube indicating the growth of microorganisms. LDPE piece from the test system was observed on the 15th and 30th day of incubation by staining with 0.5% safranine (to observe any colonization of microbes, that may otherwise get unnoticed due to the hazy nature of plastic) and wet mount observation. The experiment revealed that, although there were no signs of degradation, 'microbial film' could be spotted on the plastic piece, area of which increased by the 30th day. Biofilms are known to play crucial roles in biological processes. Since LDPE was the only source of carbon and energy in the cultivation media provided, presence of biofilm on the LDPE film indicated possible role of LDPE degrading bacteria. The formations of biofilms, however, could also be an indication of microbial growth and not of a positive plastic degradation ability of microbes. Hence, further confirmation is required.

Isolation of Gut-microbiota on dilute nutrient media

It is a very well-known fact, that the nutrient concentrations in the commonly used microbial cultivation media are several-fold higher than those present in their natural environments. A number of bacterial isolates have been shown to grow in extremely dilute culture media which failed to grow on conventional media. These 'oligotrophic' pool of bacteria seemed to be composed of a large number of novel and diverse species (Watve et al., 2000). Hundred-fold diluted nutrient media was used in the present study to order to isolate this 'unexplored wealth' that might be present in *Sitophilus* gut.

Sample from test and control were straked on sterile media plate and incubated at 37°C for 24 hrs. The colonies had an entire margin, creamy-white colour, smooth consistency, circular shape, they were flat and opaque.

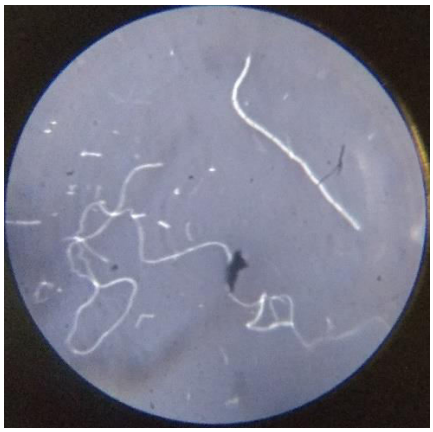

Figure 1 Negative staining of test sample with 5% nigrosine after incubation for 58 days

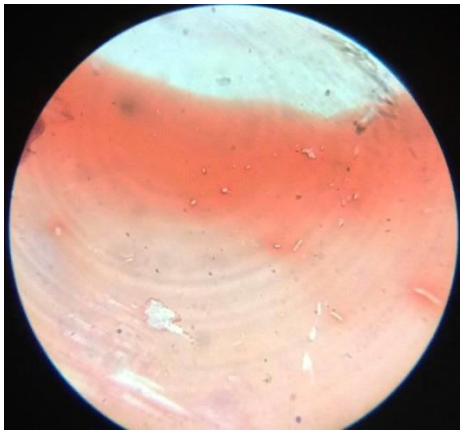

Figure 2 Cell wall staining

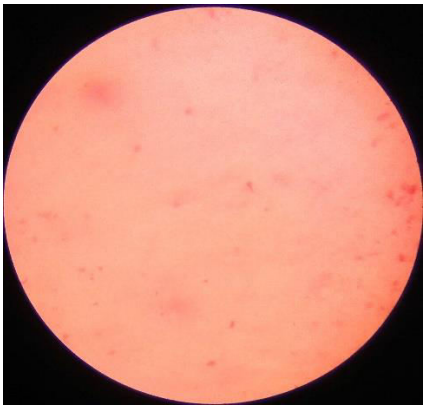

Figure 3 Stained plastic piece

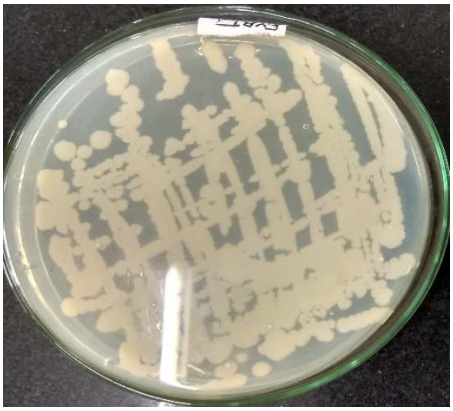

Figure 4 Colony on Nutrient agar

CONCLUSION

Current study has used "dilute" nutrient media containing 'LDPE plastic as a prime source of carbon and energy' for isolation of probable novel plastic degrading bacteria from *Sitophilus* gut. Although bacteria have been enriched and isolated, further confirmation on their plastic degrading potential by electron microscopy and FTIR is required. Molecular identification of the isolated bacteria is also needed to be performed.

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