ISOLATION AND IDENTIFICATION OF POLYETHYLENE BIODEGRADATION BACTERIAL FROM THE GUTS OF PLASTIC BAGS - EATING DAMP WOOD TERMITES

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Abstract

Plastics are used in many purposes including packaging, disposable diaper backing polyethylenes carry bags. Polyethylene (PE) bag has been considered non-biodegradable for decades. Although, the biodegradation of PE by bacterial cultures has been occasionally described, valid evidence of PE biodegradation has remained limited in the literature and first evidence termites feeding. We found that primitive termites (dry wood termites) were capable of chewing and eating Polyethylene carry bag. The present study was plastics degradation bacteria were isolated from termite gut bacteria. Two bacterial strains capable of degrading PE carry bag were isolated from this primitive termite’s gut; Lysinibacillus and Bacillus sp. T2 were identified by morphological, biochemical characterization, 16S rRNA sequencing and physical analysis of Polyethylene bag was done by SEM. The biodegradation efficacy of Lysinibacillus and Bacillus sp. T2 by using polythene carry bag were studied. The Lysinibacillus was found to be more effective in degradation of polythene bag at 30 days. Two different bacterial strains were isolated among those, Lysinibacillus (25.03 %) recorded maximum weight loss were observed followed by Bacillus sp. T2 (24.03) were estimated. An increase in incubation period there is a dramatic increase in weight loose of polythene carry bags. The results established the presence of PE-degrading bacteria in the guts of termite and provided promising evidence for the biodegradation of Polyethylene bag in the environment.

1. Introduction

One of the major environmental threats is the least rate of degradation or non biodegradability of the organic materials under natural condition, e.g. plastics. The plastics of various forms such as nylon, polycarbonate, polyethylene - terephthalate, polyethylene, polypropylene, polystyrene, polytetraflouro ethylene, polyurethane and polyvinyl chloride (Smith, 1964) are being continuously used in our day-to-day life. Polyethylene (PE), the most common petroleum-based plastic was expressed as “[CH₂–CH₂]n” and was widely used in everyday life, with an annual global production of approximately 140 million tons (Jun Yang et al., 2014). The widely used packaging plastic (mainly polythene) constitutes about 10 % of the total municipal waste generated around the globe (Barnes et al., 2009). Only a fraction of this polythene waste was recycled whereas most of the wastes enter into the landfills and take hundreds of years to degrade (Lederberg, 2000; Moore, 2008). The use of polythene was increasing every day

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and its degradation was becoming a great challenge. One of the major environmental threats is the least rate of degradation or non biodegradability of the organic materials under natural condition, e.g. plastics. The plastics of various forms such as nylon, polycarbonate, polyethylene - terephthalate, polyethylene, polypropylene, polystyrene, polytetrafluoro ethylene, polyurethane and polyvinyl chloride (Smith, 1964) are being continuously used in our day-to-day life. Polythene constitutes 64 % of the total synthetic plastic as it is being used in huge quantity for the manufacture of bottles, carry bags, disposable articles, garbage containers, margarine tubs, milk jugs and water pipes (Lee et al., 1991).

Polythene bags are made of polyethylene. The synthetic polymers are high hydrophobic level and high molecular weight. Annually 500 billion to 1 trillion polythene bags are being used routinely all over the world (Ariba Begum et al., 2015). In contrast, almost no biodegradation of PE through the biological activities of select microorganisms can be observed without pretreatments (Albertsson et al., 1987). The poor biodegradation of virgin PE has been attributed to the lack of specific plastic - degrading microorganisms with long carbon chain oxidization and depolymerization capabilities. Certain researchers have assumed that the time for microbial evolution, which was required for plastic degradation, might be much longer than the history of PE applications. To date, researchers are still attempting to discover PE - degrading microorganisms through tests involving plastic-waste-contaminated soils, landfills, compost, and marine life and have isolated several bacterial strains (Andrady, 1994). Some of these bacterial strains have shown a moderate ability to use PE as a carbon source based on the determination weight loss. For example, it has been reported that a Lysinibacillus bacterial strain causes weight losses of up to 29.5 % in the tested LDPE within 126 days, Atefeh Esmaeili et al. (2013).

Additionally, Ariba Begumhas et al. (2015) reported the isolation of two bacterial strains that rapidly degrade 20.1 % of the tested LDPE weight over 30 days. Microorganisms play a significant role in the biological decomposition of material (Shah et al., 2008). Microorganisms can degrade plastic over 90 genera, from bacteria and fungi, among them; Bacillus megaterium, Pseudomonas sp., Azotobacter, Ralstonia eutropha, Halomonas sp., etc. (Chee et al., 2010). Microorganisms, such as bacteria, fungi and actinomycetes are having capable to degrade of both natural and synthetic plastics (Gu et al., 2000) by the action of enzymes, chemical degradation with living organisms. Biodegradation resulting from the utilization of polyethylene as nutrient may be more efficient if the degrading microorganism forms a biofilm on the polyethylene surface. The primary mechanism for the biodegradation of high molecular weight polymer is the oxidation or hydrolysis by enzyme to create functional groups that improves its hydrophilicity (Huang et al., 2010) when a plastic degrades, these polymer bonds break. In this study, with Polyethylene carry bag as a sole carbon source, we isolated two Polyethylene - degrading bacterial strains from the enrichment of termite gut contents and identified that these bacteria were capable of degrading Polyethylene (PE) carry bag within a limited incubation period based on the characterization of low density formation, changes in the PE’s physical properties (tensile strength and surface topography), chemical structure (hydrophobicity and appearance of carbonyl groups), molecular weight (accompanied by the formation of daughter products), and weight loss.

Biodegradability was evaluated by weight loss, tensile strength loss, changes in percent elongation and changes in polyethylene molecular weight distribution. We found that the Damp wood termites can damage LDPE bags by chewing and eating the bag. Riudavets et al. (2007) has been previously reported similar observation stating that various species of stored - product insect pests like to chew and penetrate PE packaging films. Recently, biodegradation of PE by the two gut bacteria isolated from wax worms and indicated that the bacteria from these plastic - chewing insect larvae were a promising source of
plastic degradation microorganisms (Jun Yang et al., 2014). In the present investigation, LDPE biodegradation by the two gut bacteria isolated from termite and designated that the bacteria from these plastic-chewing termites were a capable source of plastic-degrading microorganisms.

2. Materials and methods

Collection of termites

Our university surrounded by fertile agriculture lands and they were cultivating different verities of economically important commercial crops, herbs along with number of coconut tree and fruit orchids. During the rainy seasons longs of dead wood lane’s from the cultivation land and from yard. Apart from this get concern on logs from the coconut tree and coconut leaves that fall on the ground and encumbered with termites due to the moister nature of the soil and begin to decay due to the action of soil microbes, termites and associated micro biota. The analyses of decaying log woods noted and, isolated for plating to unravel the nature of decaying period of particles and they were discussed.

Isolation of termites guts bacteria

Termites were washed with sterile distilled water, dried on a filter paper and externally sterilized with 70% ethanol. The entire gut was removed with sterile forceps and crushed. The guts were homogenized in 1 ml of sterile distilled water, serially diluted to remove large gut debris. 0.1 ml aliquot of various dilutions (10^{-3} to 10^{-5}) was spread on Nutrient agar medium (Hi-media) by using L-rod and incubation at 37 °C for 24 hrs. After the incubation period, colonies have been counted by CFU (colony forming unit) and colony morphology was observed.

Biochemical tests

Each isolate was tested for its ability to grow in anaerobic condition by performing the OF test (Oxidative-fermentative) as described by Hugh and Leifson (1953). Gram straining, indole, methyl, VP, citrate utilization and starch hydrolysis isolates as described by Murinda et al. (2002).

Polyethylene degradation of termite guts bacteria

Physical analysis SEM

The surface morphology of the PE film was analyzed through Scanning Electron Microscopy to check for any structural changes on the film. A piece of film was placed on the sample holder and was scanned at a magnification of 17000 x, 28000 x, 40000 x, 50000 x and 60000 x (Ikada, 1999).

Identification of bacteria by sequencing of the 16S rRNA gene

Genomic DNA Extraction

The genomic DNA was extracted from the isolated bacterial colony using a protocol modified from salting out procedure described by Kannan et al. (2015). The overnight bacterial cultures were centrifuged at 10,000 rpm at room temperature (RT) for 5 minutes. The pellet was mixed with 300 μl of HLB consisted of 10 % SDS (w/v), 1 M Tris-HCl, 0.5 % of EDTA and 4 M NaCl, after mixing The mixture was incubated with RNase (10 mg/ml) for 5 minutes. Then, 100 μl of saturated 6 M NaCl and 200 μl of Chloroform was added and shaken vigorously for 30 seconds, followed by centrifugation for 10,000 RPM for 10 minutes. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. An equal volume of absolute ethanol was added carefully down the wall of the tube. The mixture was centrifuged again at 10,000 RPM for 5 min and the supernatant discarded. Subsequently, the precipitated DNA was a pipette out to a new Eppendorf tube. An equal volume of absolute ethanol was added carefully down the wall of the tube. The mixture was centrifuged again at 10,000 RPM for 5 min and the supernatant discarded. Subsequently, the precipitated DNA was a pipette out to a new Eppendorf tube and washed twice with 70 % ethanol by centrifugation at 5000 RPM for 5 minutes. The pellet was air dried at room temperature for 10 minutes. Finally, the pellets were suspended in 50 μl of TE buffer. The DNA sample was separated according to their molecular weight under electrophoresis system. Finally, the DNA band was visualized under Gel Documentation system (Lark, Germany). The
DNA concentration was determined by measuring the absorbance at the ration 260/280 nm and the
DNA suspension was stored at -85 °C until it was
used for PCR and further analysis.

The isolates were identified by 16S rRNA
gene sequence; it was amplified by polymerase
chain reaction (Kolbert and Persing, 1999). The
PCR reaction volume (50 μL) containing 25 μL of
2X Prime Taq, 1 μL both primers (5’-AGA GTT
TGA TCM TGG CTC AG - 3’) (5’- AAG GAG
GTG ATC CAN CCR CA - 3’), 21 μL of Milli Q
water and 2 μL of extracted DNA as a template in
0.2 ml thin walled PCR tube, the PCR product was
purified and sequenced at Eurofins Genomics,
Bangalore, India. The sequences were compared
to sequences in the public database with Blast
search program on the National Center for
Biotechnology Information (NCBI) website
(http:/www.ncbi.nlm.nih.gov) to find closely
related bacterial 16S rRNA sequences. Phylogenetic trees were calculated according to
the neighbor joining method (Saitou and Nei,
1987) and maximum-likelihood (Felsenstein,
1981), and visualized using tree view of the same
software. Sequence similarity matrices were
calculated.

Biodegradation of polythene carry bag

The degradation of by bacteria was studied
by following the method of Kathiresan (2003).

Surface sterilization of polythene carry bag

The collected plastics bags were cut into
small pieces and cleaned with tap water and
surface sterilized with ethanol. Then washed with
distilled water, 0.1 % mercuric chloride and again
washed with distilled water.

Degradation of polythene carry bag

Nutrient broth was prepared and autoclaved at 121 °C for 15 minutes. A volume of
200 ml of cooled, nutrient broth was poured into
eight 250 ml sterile conical flasks. The sterile pre
weighed polythene bag pieces were aseptically
transferred into nutrient broth. A loopful of
bacterial cultures such as Bacillus sp. and
Lysinibacillus was inoculated into nutrient broth.
One 250 ml of flask containing the polythene bag
pieces without bacterial cultures was maintained
as control. These flasks were incubated at 37 °C
for 10, 20 and 30 days. The polythene bag pieces
were carefully removed from the culture (by using
forceps) after different days of incubation. The
collected pieces were washed thoroughly with tap
water, ethanol and then distilled water. The pieces
were shade dried and weighed for final weight.
The data were recorded. The same procedure was
also repeated for all the treated samples.

Determination of degradation of polythene
carry bag

The percentage of degradation of polythene bag pieces by Bacillus sp. and
Lysinibacillus was determined by calculating the
percentage of weight loss of plastics. The percentage of weight loss was calculating by the
following formula.

Percentage of weight loss

\[
\text{Percentage of weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100
\]

3. Results and Discussion

Two bacteria were isolated during the
improvement gut contents of the LDPE-eating
damp wood termite when using LDPE as a sole
carbon source. The isolates were purified and two
polyethylene bacterial strains ultimately degraded
the provided LDPE (2.5 × 2.5cm sq) and the
minimal carbon free broth was prepared the LDPE
(Low Density polyethylene) films. In the present
investigation, two bacteria were isolated from
termite gut bacteria and identified its
morphological and biochemical characterization.
The results were summarized in Table - 1. The
bacterial isolates were Bacillus sp. and
Lysinibacillus. The results were comparable with
the earlier finding of Kathiresan (2003); Atefeh
Esmaeili (2013); Jun Yang (2014) and Ariba
Fig - 1: Termites feeding carry bags with three plastic degradation bacteria

Fig – 2: SEM images of degraded PE films - *Bacillus* sp.

Fig – 3: SEM images of degraded PE films - *Lysinibacillus*
Table - 1: Physiological and biochemical characteristics of polyethylene degrading bacterial

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Bacillus cereus</th>
<th>Lysinibacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Morphology</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>V-P test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>OF Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSI</td>
<td>Acid/Alk</td>
<td>Acid/Alk</td>
</tr>
</tbody>
</table>

Table – 2: Biodegradation of polythene carry bag by Bacillus sp.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Initial weight of polythene carry bag (g)</th>
<th>10 days</th>
<th>20 days</th>
<th>30 days</th>
<th>% of weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
<td>1.000</td>
<td>0.8780</td>
<td>0.8120</td>
<td>0.7530</td>
<td>24.7</td>
</tr>
</tbody>
</table>

Table - 3: Biodegradation of polythene carry bag by Lysinibacillus

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Initial weight of polythene carry bag (g)</th>
<th>10 days</th>
<th>20 days</th>
<th>30 days</th>
<th>% of weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysinibacillus</td>
<td>1.000</td>
<td>0.8590</td>
<td>0.8010</td>
<td>0.7220</td>
<td>27.8</td>
</tr>
</tbody>
</table>

Fig – 4: Phylogenetic tree result of Bacillus sp. strain using Clustalw software
Physiological Analysis SEM of Polyethylene bag

Structural changes and erosions on the surface of the polythene bag were observed. Cavities were also observed on the polyethylene surface. After 30 days incubation period, the physical change in surface was examined using SEM. Also, the microbial propagation has been initiated from these cracks. Such colonization and adhesion by microorganisms are a fundamental prerequisite for biodegradation of the polymer. Cavities were also observed on the polycarbonate surface (Artham and Doble. 2008).

Similarly in the present study, the images of Scanning Electron Microscopy (SEM) showed bacteria colonizing over the film. Also, cavities were observed in the film initiating biodegradation of the polymer.

Most of the bacterial strains of isolates have been rod shaped formed pairs and chain, and other cultural physiological and biochemical characters were identified and shown in Table - 1. The OF test (Oxidative fermentative) for these species were positive, which indicates their ability to grow in both aerobic and anaerobic condition. All bacterial cells Gram, rod shaped, facultative

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closest match in GenBank</th>
<th>Closest match in GenBank with accession number</th>
<th>Homology (%)</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
<td>Bacillus cereus strain 45-7 16S rRNA gene, partial sequence</td>
<td>HM104650.1</td>
<td>100 %</td>
<td>0.0</td>
</tr>
<tr>
<td>Lysinibacillus</td>
<td>Lysinibacillus macroides strain AB-71 16S rRNA gene, partial sequence</td>
<td>KT027760.1</td>
<td>100 %</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Fig - 5: Phylogenetic tree of Lysinibacillus created by using Clustal w neighbor-joining tree

Table – 4: GenBank Database homology of the bacterial isolates from damp wood termites
anaerobe and motile based on the Bergey’s manual Brenner et al. (2005). The Bacillus sp. bacterial cells were Gram - positive, rod shaped, facultative anaerobe and motile and several Bacillus species identified have been isolates from the termite was species were Bacillus sp. termite gut (Kutnigh et al., 1994). Bacillus sp. has been isolated from the Niger Delta mangrove swamp and soils are capable of growing have potential for used in biodegradation of PE. They were identified by the fact that they were aerobic, rod-shaped and Gram - positive cells. Lysinibacillus is Gram - positive, rod - shaped, and round spore forming bacterial genus of the family Bacillaceae. Many bacterial species were isolated from the digestive tracts of the woodborers belong to the genus Lysinibacillus (Bosire Carren et al., 2013).

Identification of the polyethylene degradable bacteria

Two different samples were identified by micro and macroscopically and confirmed by various biochemical tests. The 16S rDNA sequences were matched for local alignment through NCBI-BLAST. Based on the Gram staining, Colony morphology and Biochemical test (Table - 1), two strains were identified (Soni et al., 2010). In this present study, isolation identified strains were Bacillus sp. and Lysinibacillus were identified through 16S rRNA genomic sequence analysis and submitted in NCBI.

16S rRNA gene sequencing

The 16S rRNA gene was amplified using 27f and 1492R primers using the genomic DNA isolated from Bacillus sp. and Lysinibacillus by PCR which resulted in the amplification of expected amplicon of 1500 bp. Homology analysis of the sequences showed 100 % homology with the available 16S rRNA sequences in the GenBank (Table - 3). Among the two isolates, the 16S rRNA gene of Bacillus sp. had 100 % homology with an isolate reported from USA. Lysinibacillus had 100 % homology with UK isolates respectively, available in GenBank.

Phylogenetic analysis

The 16S rRNA gene sequence of the isolates revealed that isolates Bacillus sp. (T2) was closely related to Bacillus sp. with 100 % sequence similarity (Fig - 4), isolated in Lysinibacillus with 100 % sequence similarity (Fig - 5).

Acknowledgement

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4. Reference

