Studies on Biodegradation of Polyethylene terephthalate: A synthetic polymer

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ABSTRACT

The degradation of polyethylene terephthalate (transparency sheets) by microbes and esterase was studied. Important chemical changes of polymeric chains were detected by X-ray photoelectron spectroscopy (XPS) analysis. After the microscopic and transmission electron microscopy (TEM) studies, specific microbial media was used to isolate and identify the bacteria from the degrading polyethylene terephthalate. Degradation impact by microbes on the crystalline structure and presence of microbes inside the polyethylene terephthalate were seen in scanning electron microscopy (SEM) micrographs. A record of loss in weight and tensile strength were also made. Esterase was found to be involved in the biodegradation. Although the biodegradation was slow and weak, we demonstrated with chemical and x-ray diffraction analysis (XRD) as well as with SEM micrographs that microbes and esterase could act on the polyethylene terephthalate. The microbe involved was confirmed to be a Nocardia species.

Keywords: Polyethylene terephthalate, Nocardia, Bacillus subtilis, biodegradation, esterase.

INTRODUCTION

The ecological problems related to the environmental pollution by synthetic polymers like plastics are one of the major concerns of the present days; especially because they are difficult to degrade easily and the entire process is time consuming. The environmental concerns include air, water and soil pollution. Latest research has also suggested that polyethylene terephthalate might yield endocrine disruptors under conditions of common use [1]. Different polymers like polyethylene, polypropylene, polyvinyl chloride, polystyrene, polyester, polyamide, polyethylene terephthalate, polyolefin and polyurethane [2] have been designed for resistance to the environment. As a result they undergo degradation or bio-degradation, at very slow rate. Thermal degradation is another approach, however it is even more hazardous as it generates toxic gases like carbon monoxide and dioxin, and burning of plastic has shown to release heavy metals like cadmium and lead. In an attempt to conserve energy and minimize waste, research towards producing biodegradable polymers using starch is in effect. It has been proved [3] that microbes like Phanerochaete chrysosporium, Trametes versicolor, Streptomyces species, a Aspergillus niger, Pleurotus ostentus, Geophyllum trabeaum, Thermonospora and Actinomadura have the ability to degrade polymers. Later it was attempted to prepare biodegradable co-polymer or bioplastics using a Pseudomonas strain [4]. Active research in the area of manufacturing biodegradable plastics that will decompose in natural aerobic (composting) and anaerobic (landfill) environments are also in effect [5]. Under regulated conditions biodegradable plastics could potentially degrade to the point where microorganisms can metabolize them.

In the present work attention was focussed on the active role of microbes present in the environment in biodegrading a strongly bonded polymer, when a roll of transparency sheet kept in a closed drawer untouched for nearly a year; was found to be degrading. Although the transparency sheet was kept away from light and dust, the possibility of
soil microbes reaching these sheets or a probable bacterial mutation could not be ruled out. The sheet was brittle at places, had white coloured microbes growing on it along with a foul smell too. This transparency sheet was polyester, (polyethylene terephthalate Fig 1); hence possibility of secretion of esterase by the microbe for biodegradation has also been looked into. Packaging manufacturers use polyethylene terephthalate plastic mainly because of its strength, thermo-stability and transparency. We have attempted to isolate and identify the microbes that were seen growing on the polyethylene terephthalate sheet - possibly degrading it, and studying the physical and chemical impact of degradation.

![Molecular structure of Polyethylene-terephthalate (C₆H₄O₂)n](image)

**Figure – 1: Molecular structure of Polyethylene-terephthalate (C₆H₄O₂)n**

**MATERIALS AND METHODS**

The objective of the present work was identification of the microorganisms that was responsible for degradation of the transparency sheet. Was the degradation caused by a single microbe or was it the action of multiple microbes? To achieve this end, preliminary studies were conducted making a record of visual observations, usage of a light microscope followed by in depth analysis using a sophisticated scanning electron microscope was performed, where data on the degradation of polyethylene terephthalate was recorded. This was followed by a systematic study to isolate and identify the microbes.

**Optical microscopic observation:** The infected transparency sheets as well as microbial cultures were examined at all the stages under a Reichert Jung Microscope and photographed using Leitz microscope with an attached camera.

**Scanning electron microscopy:** SEM observations were accomplished by loading the sample on to the Strobe in ESEM FEI QUANTA 200 microscope. 15KV was applied to the sample at Tilt 0.20 and an amplification of AmpT 10.0. SEM images were taken at 6000X resolution.

**X-ray diffraction pattern of transparency before and after degradation:** X-ray diffraction (XRD) was performed using Jeol – JDX 830, with Cu - Ka R-ray machine.

**Isolation of fungi:** In order to isolate fungi from the degraded sheets different fungal growth media were used. Specifically, Saboaurds agar, Potato dextrose agar (PDA) and a complex medium consisting of malt extract, glucose yeast extract and peptone agar. 20 ml of respective media was poured into test tubes and slants were prepared after sterilizing it at 121°C for 30 min. Infected plastic sheets were cut into 1 cm² pieces and inoculated onto the test medium. Cultures were incubated at 10, 25 and 35°C ± 1°C in dark as well as light (3000 Lux) conditions. Growth of fungi was recorded at regular intervals.

**Isolation of bacteria and actinomycetes:** The selection of media to grow bacteria and actinomycetes were based on the SEM and light microscope observations with the help of Bergey’s manual. The media tried were Actinomycetes Isolation Agar (AIA), Brain Heart Infusing Agar (BHIA), Glycerol Asparagine Agar Base (GAAB), Alternat Thioglycolate Broth (ATGB), Bushnell and Hass Broth (BHB), Alkaline Czapeck Broth (ACB), Luria-Bertani medium (LB plates) and Luria Bertani media containing Tributyryl (LBT). 20 ml of respective media was poured into test tubes and slants were prepared after sterilizing it at 121°C for 30 min. Infected plastic sheets were cut into 1 cm² pieces and inoculated onto the test medium. Cultures were incubated at 10, 25 and 35°C ± 1°C in dark and light (3000 Lux) conditions. Growth was recorded at regular intervals.

**Fungal and bacterial staining:** Lacto-phenol cotton blue was used to stain the fungi where as bacteria were stained using the Gram stain.

**Effect of microbes on the physical properties of polyethylene terephthalate** - Isolated microbes were inoculated on to fresh (control) 1cm² sheets of transparency sheet on the corresponding medium from which they were isolated. The following physical parameters studied.

**Loss in Weight** - Fresh transparency sheets of 1cm² were weighed after sterilizing them with 1% hypochlorite and quick dip in alcohol. The isolated microbes were then inoculated onto the fresh (control) 1cm² sheets of

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transparency sheet in respective medium from which they were isolated and grown. Change in weight of the transparency was recorded on day 20, 40, 60, 80, 100, 120, 140, 160 180 and 200.

**Change in tensile strength of microbe treated transparencies** - Transparency sheets were cut in the shape of a dumb-bell with the help of a cutting-die. The dumb-bell shaped transparency had the measurements as shown in figure 2. Tensile strength of the transparency was measured prior to inoculating them along with microbes and 200 days after growing them with microbes. The dumb-bell shaped transparency was placed between the jaws of tensile strength testing machine INSTRON UTM 1145. One jaw was fixed and the other moving jaw was moved at the rate of 60 mm/minute. The load at break and the ultimate elongation was measured. Loss in tensile strength was measured using the following formula

\[
\text{Tensile Strength (Pa) = \frac{\text{Load (Kg) x 9.8}}{\text{Width (mm) x Thickness (mm)}}}
\]

Percentage of elongation was calculated by the following formula:

\[
\% \text{ Elongation } = \frac{\text{Elongation (cm) x 100}}{\text{Initial length (cm)}}
\]

**Figure – 2: Shape and dimensions of polyethylene terephthalate used for measuring the tensile strength**

**Effect of microbes on the biochemical properties of polyethylene terephthalate:** Isolated microbes were inoculated on to fresh (control) 1 cm² sheets of transparency sheet in the corresponding media from which they were isolated. It was envisaged that the degradation of the polyester-polyethylene terephthalate or the transparency was due to some extra-cellular leachates secreted by the microbes present on the sheet. Initial studies were conducted to analyze probable presence of esterase, an ester degrading enzyme. On hydrolysis ester produces an acid and an alcohol apart from water, CO₂, CO and H₂. Hence, subsequently analysis of acid content and esterase activity as an indicator for measuring the degradation of polyethylene terephthalate was performed.

**Acid estimation:** Acid content was estimated in three sets of samples: control (media only), blank (ester solution i.e. ethyl acetate without any media) and an experimental. The experimental sample had 1 cm² of solid media or 2 ml liquid media in case of broth that was obtained by centrifuging at 2500 rpm for 15 min. To both solid and liquid media 10 ml of ester solution was added. The acid inherently present in the ester solution was determined and considered as the blank reading. This was obtained by diluting 0.2 ml of pure ethyl acetate in 8ml of deionised water followed by titration against 0.01N NaOH (which required 0.11 ml of 0.01N NaOH). Thus the acid concentration present in the ester solution was calculated to be 0.13 x 10⁻³N. We proceeded with the further calculations based on the above result that 0.13 x 10⁻³N of acid is inherently present in the ester solution used for the hydrolysis experiment of various culture media. Hence, in the actual calculations the above equivalent was subtracted from all the experimental results. To test the presence of acid in the ester hydrolyzed by the microbes, in the respective media, titrimetric analysis of leachates was performed. Leachates from both solid and liquid media were allowed to diffuse through the ester solution for different time periods. After 1st, 2nd, 4th, 6th and 8th day, 2 ml aliquots were diluted to 10 ml using autoclaved HPLC grade water. Three drops of 1% alcoholic phenolphthalein was added to it and the solution was then titrated against 0.01N NaOH. The amount of NaOH required to hydrolyse the ester by microbes from various media was recorded and concentration of the acid was calculated by using following formula:
\[ N_1 \times V_1 = N_2, V_2 \]

where \( V_1 \) = volume of diluted acid obtained by hydrolysis of ester after addition of 8ml distilled water. Final volume was 8.2 ml (including 0.2 ml of hydrolysed ester)

\( N_1 \) = concentration of acid (Normal) under consideration

\( V_1 \) = volume of alkali (NaOH) required to neutralize the acid

\( V_2 \) = concentration of alkali (i.e. 0.01N NaOH)

Similarly, concentration of the acid was calculated for control and blank samples also.

**Esterase analyses:** To confirm that the microbes were responsible for degradation of the transparency sheet, the presence of the enzyme esterase was tested. Those microbes which grew on the respective media as well as confirmed the presence of an acid were further analysed to test for the presence of esterase. Briefly, bacteria were subcultured on Luria Bertani plates and allowed to grow at 30 and 35°C. After 72 hours, when an appreciable amount of bacteria grew, they were subcultured on Luria Bertani media containing tributyrin and further grown at 30 and 35°C in the dark. Presence of esterase is confirmed by formation of a clear zone around a bacterial colony.

**RESULTS AND DISCUSSION**

**Visual observation of the transparency:** Visual observation revealed a remarkable brownish white discolouration of the sheet. It appeared very brittle and crumbled at places. There was a distinct foul smell that persisted around the sheet, which could be due to the aromatic ring of the monomer that degraded. A white powder like substance was also seen on the sheet, presumed to be microbes or some chemical (Fig 3a).

**Optical microscopic observation:** Figure 3b depicts disorientation of the polyethylene terephthalate crystals. Polyethylene terephthalate is known to exist both as an amorphous (transparent) as well as a semi-crystalline polymer. Similar observations were made earlier [3] demonstrated with SEM micrographs that microbes could act on the polyethylene terephthalate and develop some cracks on the surface. Though recently it has been suggested [6] the biodegradation of polyethylene terephthalate by *Penicillium funiculosum*; none of the sheets showed uptake of Lacto-phenol cotton blue stain, thus ruling out the presence of fungi. However, uptake of Gram positive and Gram negative stain was observed under optical microscope, confirming the presence of bacteria.

**Scanning electron microscopy:** SEM micrographs of the degrading polyethylene terephthalate showed crystals of degraded product as well as microbial colony growing inside as well as on the surface of the crystals (Fig 3c). Presence of abundant colonies of branched mycelium having a width of 0.1µ to 0.25µ throughout the infected transparency sheet (fig 3d, 3e) was clearly visible. This indicated presence of Actinomycetes. Accordingly a specific media that is suitable for the growth of Actinomycetes was used to isolate it. Some samples also showed the presence of rod shaped bacteria of 1µ length and 0.25 to 0.5µ length (fig 3f).

It has been shown that *Bacillus species* is involved in initial degradation of dimethylphthalate by esterases [7].

Hence isolation of Bacillus was also done. This observation suggests presence of more than one type of microbe on the transparency sheets. However, it does not confirm that all microbes may be involved in degradation of polymer. Modifications of surface chemistry of polyethylene terephthalate by many marine bacteria have also been observed [8].

**X-ray diffraction studies:** As expected XRD spectrum (figure 4) of the non-degraded transparency sheet appeared to have a non-crystalline polymer nature, having two broad peaks (one large and one small). However, XRD of the degraded transparency sheet showed the presence of many peaks (2θ) suggesting the presence of a crystalline material. The presence of crystalline structures in the degraded polymer may be the reason for appearance of white powder like substance on the surface of degrading transparency sheet. These (2θ) values were matched with the XRD of the published phthalate related compounds. By matching the values available in ICPDS (ASTM), the XRD of the degraded polyethylene terephthalate revealed the presence of three types of phthalate derivatives i.e.

1. 3-amino-phthalic acid hydrochloride (C₆H₄ClNO₄) [PDF/JCP No 19-1534]
2. Phthalamic acid phthalic acid mono-amide (C₆H₇NO₃) [PDF/JCP No 33-1800] and
3. tetra-hydrophthalic anhydride (C₆H₈O₃) [PDF/JCP No 35-3891]

It could be argued here that the degradation of the transparency sheet caused by microbes occurred to various chemical stages of the polymer thus resulting into different types of compounds or the transparency sheet being a...
terepolymer (having three types of monomers in the polymer chain) might have degraded into three types of different derivatives of polyethylene terephthalate ester. Moreover, the involvement of microbes may account for presence of amino acids and amide groups in the degraded product of the polymer. In polyethylene terephthalate monomers are bonded via ester linkages and enzymes that degrade those by hydrolysis are esterase, an enzyme that is ubiquitous in living organisms. Nevertheless several biodegradable plastics are made by using bacterial polyesters (polyhydroxyalkanoates).

Figure – 3 Visual and microscopic observation of degrading polyethylene terephthalate showing (a) A piece of degraded sheet (b) Disoriented crystal structure of polyethylene terephthalate as seen under light microscope. SEM image showing (c) A degrading crystal ‘c’ and microbe ‘m’ inside as well as outside the crystal (d) Branched mycelial microbe on the surface of a crystal (e) Close up of a microbe with parts of degraded polyethylene terephthalate and (f) Rod-shaped bacteria found on the surface of the sheet.
Growth of fungi on different media: Fungus did not grow on any of the three fungal growth media, confirming that fungi are not involved in degradation of the transparency sheet. Moreover staining with fungus specific stain also did not show presence of any fungi.

Growth of bacteria on different media: As mentioned earlier in the materials and methods five different types of media (AIA, BHIA, GAAB, ATGB and BHB) that are suitable for growth of both actinomycetes and bacteria were tried. Impact of temperature and light were also studied. As seen in Table 1, microbes grew on all the three tried solid media (Fig 5a, b, c) and 2 liquid media (Fig 5d, e). The most suitable temperature for the growth was 35°C. Microbes grew in both light and dark; however, the growth was better in the dark.

Different types of bacteria grew on the tested media. Elongated wrinkled greyish colony grew on AIA medium (Fig 5a), which could be a soil actinomycetes. Two types of microbes grew on BHIA media: a large off white colony with filamentous microbes and a small transparent colony having 

Table -1: Growth of microbes in five different media under different physical parameters (temperature and light/dark conditions) [- = no growth, +_ =  good growth and +++ = very good growth]

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth in light at 10°C</th>
<th>Growth in light at 25°C</th>
<th>Growth in light at 35°C</th>
<th>Growth in dark at 10°C</th>
<th>Growth in dark at 25°C</th>
<th>Growth in dark at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIA</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BHIA</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>GAAB</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>ATGB</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>BHB</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

All the above mentioned microbes that were isolated on different media were later grown on the control (uninfected) transparency sheets to see which of them could degrade the polymer. The impact on physical property of transparency as well as chemical changes was also recorded.
Figure – 5: Growth of bacteria from degrading polyethylene terephthalate sheet on (a) Actinomycetes Isolation Agar (AIA), (b) Brain Heart Infusing Agar (BHIA), (c) Glycerol Asparagine Agar Base (GAAB), (d) Alternate Thio-glycolate Broth (ATGB) and (e) Bushnell and Hass Broth (BHB) media.

Figure – 6: Microbes grown on various media and their effect on weight loss of transparency sheet. Days indicate the period for which transparency sheets were exposed to the microbes.

Weight loss: The bacteria that were grown on various culture media had good source of nutrients, however it must be noted here that the microbes that grew on transparency only had the polyester as the source of carbon. Therefore, to study the effect of these microbes on weight of the transparency it was necessary to grow them on a media devoid of any other carbon source other than the polyester itself. As it was difficult to prepare any of the above mentioned
media without carbon, we thus tried alkaline Czapek broth (AC) (Table 2). This media is suitable for growth of many soil bacteria, saprophytic fungi and many other microbes. Fresh transparency sheets of 1cm² were weighed and inoculated in AC media and broth. As seen in figure 6, maximum loss of 8% in weight on 200th day was recorded when the transparency sheet was kept in contact with the microbe isolated using BHB medium. Decrease on weight of the transparency followed the pattern ATGB>GAAB>AIA. However, no loss in weight was recorded in presence of microbes isolated on BHIA medium. Microbes from BHB medium showed decrease in weight (0.2%) right from the 20th day and steadily continued till 200th day (Fig 6).

**Change in tensile strength:** Table 2 shows change in tensile strength and elongation of the transparency sheet exposed to microbes isolated from different media, on 200th day. As expected noticeable loss in tensile strength was recorded only when the microbes isolated in the BHB medium was used. Microbes from ATGB medium showed slight loss in tensile strength.

**Table 2:** Loss in tensile strength (Pa) and elongation of polyethylene terephthalate sheet kept in contact of microbes (isolated from degrading sheet) after 200 days

<table>
<thead>
<tr>
<th>Media</th>
<th>Tensile strength (Pa)</th>
<th>Elongation percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>182.0</td>
<td>4.76</td>
</tr>
<tr>
<td>Control</td>
<td>182.0</td>
<td>4.76</td>
</tr>
<tr>
<td>AIA</td>
<td>182.0</td>
<td>4.76</td>
</tr>
<tr>
<td>BHIA</td>
<td>182.0</td>
<td>4.76</td>
</tr>
<tr>
<td>GAAB</td>
<td>181.4</td>
<td>4.76</td>
</tr>
<tr>
<td>ATGB</td>
<td>180.0</td>
<td>4.76</td>
</tr>
<tr>
<td>BHB</td>
<td>176.0</td>
<td>4.76</td>
</tr>
</tbody>
</table>

**Titrimetric estimation of acid:** Presence of acid in the ester solution treated with the medium with and without the microbes was performed to analyse the chemical change due to microbial leachates during degradation of transparency sheet. The results after calculations (Table 3) clearly show presence of higher amount of acid in ATGB and BHB medium. In BHB it was two-fold higher than ATGB media. Microbes that grew in BHB medium were found to cause maximum loss in transparency weight and tensile strength also. These results prompted further analysis of the enzyme esterase present in microbes grown on BHB medium.

**Table -3:** Acid content in hydrolyzed polyethylene terephthalate before and after treatment with microbes isolated from and grown on different culture media

<table>
<thead>
<tr>
<th>Media</th>
<th>Concentration of acid in hydrolyzed ester</th>
<th>Concentration of acid in ester solution</th>
<th>Actual concentration of ester after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIA</td>
<td>0.13 x 10⁻³ N</td>
<td>0.13 x 10⁻³ N</td>
<td>0.0</td>
</tr>
<tr>
<td>BHIA</td>
<td>0.13 x 10⁻³ N</td>
<td>0.13 x 10⁻³ N</td>
<td>0.0</td>
</tr>
<tr>
<td>GAAB</td>
<td>0.13 x 10⁻³ N</td>
<td>0.13 x 10⁻³ N</td>
<td>0.0</td>
</tr>
<tr>
<td>ATGB</td>
<td>0.13 x 10⁻³ N</td>
<td>0.33 x 10⁻³ N</td>
<td>0.2 x 10⁻³ N</td>
</tr>
<tr>
<td>BHB</td>
<td>0.13 x 10⁻³ N</td>
<td>0.60 x 10⁻³ N</td>
<td>0.47 x 10⁻³ N</td>
</tr>
</tbody>
</table>

**Esterase analysis:** Microbes that grew on BHB medium were further subcultured on Luria Bertani medium at 35°C in dark. Three types of microbial colonies appeared (Fig 7a): opaque white and circular, transparent white and circular and transparent colonies with irregular edges. Two microbes were identified to be *Nocardia* and *Bacillus subtilis*. Presence of *Bacillus subtilis* was confirmed (Fig. 7b and 7c) by culturing it on Himedia starch agar (M 107) and Nocardia was confirmed by culturing them on Himedia Actinomyces agar (M 431).

Tests were carried out to analyze presence of the enzyme esterase in these three microbial colonies by sub-culturing them from Luria Bertani to Luria Bertani media containing tributyrin and growing them at 35°C in dark. Presence of esterase was confirmed in only one of the three bacterial colonies, by the presence of a clear ring around the colony (fig. 8a marked x). A micrograph of the esterase producing microbe (fig. 8b) showed branching of *Nocardia* colony. There are many microbes that can biodegrade polyesters. There are many marine bacteria that can do modifications of surface chemistry of polyethylene terephthalate [8]. Hydrolysis of di-ester phthalates by esterases isolated from *Nocardia erythropolis* has been shown [9]. Moreover, role of esterase isolated from *Comamonas acidovorans* TB-35 a bacterium, in degrading polyester polyurethane has been shown [10], though polyurethane is known to be resistant to microbial attacks.
As mentioned above one of the other two colonies on LB plate was identified as *Bacillus subtilis* and the other could not be identified. But that does not rule out the possibility of them producing other leachates like depolymerases that can break the bonds of polymers. There are many reports of biodegradation of biodegradable polymers by various enzymes [11, 12,13]; Teeraphatpornchai et al 2003). It was suggested that synthetic LC cutinase can degrade polyethylene terephthalate at much higher rate than those reported by bacterial and fungal cutinases [14, 15]. It is worth mentioning here that only microbial or fungal enzymes have been topic of interest for degrading polymers but studies have been made on enzymes of animal origin also. The gastrointestinal fluids of sea turtles can also degrade synthetic polymers [16].
Figure – 8: (a) Three types of microbes isolated on LB medium as shown in fig. 7a, when subcultured on LB + tributyrin medium: only one of them shows a clear zone around the colony confirming the secretion of esterase. (b) Esterase secreting microbe *Nocardia* as seen under microscope.

CONCLUSION

In the present work no external source of microbes or microbial enzymes were used. Naturally growing microbes on the degrading polyethylene terephthalate were studied [17]. The efforts presented in this paper confirm slow microbial degradation of polyethylene terephthalate by *Nocardia* with help of the esterase enzyme. However, it suggests the possibility of involvement of other microbes also. Moreover, important chemical changes of polymeric chains were detected by XPS analysis confirming the degradation of the polymer. Since, esterase was found to be associated with the biodegradation of polyethylene terephthalate it would be a good idea to develop a high esterase producing strain of *Nocardia* or any other microbe for the biodegradation of polyethylene terephthalate.

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