Municipal Solid Waste Characterization And Its Assessment For Fungal Bioremediation

Summary of the Thesis Submitted to
The Maharaja Sayajirao University of Baroda, Vadodara

For the degree of
Doctor of Philosophy in Botany

By
Ms. Mitu Mewada

Under the guidance of
Prof. Susy Albert

Department of Botany
Faculty of Science
The Maharaja Sayajirao University of Baroda Vadodara

SUMMARY

The growing rate of population influences the enhancement of municipal solid waste (MSW) generation. Municipal Solid Waste contains household and commercial refuse including paper, textiles, food and vegetable waste, and wood and non-degradable materials; leather, plastics, rubbers, metals, glass, and electronic waste. In this advanced society, polymers are immensely significant; being a key component in most parts of urban life, for example, garments, bundling, transportation, and correspondence. Polymers are enormous atoms comprising countless small components known as monomers. Polyethylene material has proven to be inert plastic material due to its high molecular weight, complex three-dimensional structure, and hydrophobic nature. Polymers are atoms made up of countless small monomers.

Polyethylene is the most widely used plastic on the planet, being made into products ranging from transparent food wrap and shopping bags to packaging bottles and automobile fuel tanks. It can be categorized into high-density, low-density, and linear-low-density polyethylene (HDPE, LDPE & LLDPE). LDPE is considered to be tough, resistant to chemicals & flexible, whereas HDPE is more rigid & harder and also has a greater tensile strength than LDPE (Satlewal et al., 2008). LLDPE also has higher tensile strength and higher impact and puncture resistance than LDPE material. All kinds of plastics have immensely been used during the past decade in all production sectors resulting in serious problems due to their accumulation in the environment (Sowmya et al., 2015). The efficient decomposition of plastic bags takes about 1000 years (Pramila and Vijaya Ramesh 2011; Usha et al. 2011).

Bacteria, fungi, and algae are the biological factor that degrades plastic naturally (Rutkowska et al. 2002). Decomposition or destruction of contaminant molecules by the action of the enzyme secreted by microorganisms is known as biodegradation. Microbes may be able to break down the polyethylene structure since the chemical structure of polyethylene is similar to that of linear alkanes, which are known to be biodegradable (Albertsson et al., 1987). A Summary of studies on biodegradation of plastics is given by Kale et al., (2015) in which it can be understood that there are a large number of potential fungal strains which can degrade plastics.

Though there are lots of reports demonstrating the potential of plastic-degrading microbes, they lack efficiency within the context of practical application. Thus, there is a strong need to identify efficient organisms and develop technologies capable of degrading plastic efficiently without affecting the environment. Their main feature in the bioremediation mechanism is the production of extracellular enzymes like laccases, peroxidases & esterases and directly initiate microbial attachment on the PE

surface and the consequent biodegradation (Wei and Zimmermann 2017). However, very few research studies have covered the aspect of enzyme activities responsible for the degradation of polyethylene. This research aspect aims in-depth study of the enzymes involved in this process, enhancement of the degradation, and its potentiality under natural conditions. The present study emphasizes finding the non-biodegradable component of MSW and identifying a potential fungal strain for the degradation of non-biodegradable elements, especially plastics.

Research outline:

To achieve the aim of this study, the research methodology includes the collection and characterization of municipal solid waste (MSW) of Vadodara city and identification of non-biodegradable components of MSW. Followed by this isolation and fungal diversity, screening, and selection of potential fungal isolate, evaluation, and optimization of plastic degrading enzymes, and lastly assessing the practical application of the polyethylene degradation by fungus. MSW samples were collected from temporary dumping sites and landfill areas of Vadodara city for the study of the physical characteristics of MSW, which revealed polyethylene material as a non-degradable component.

Isolation of fungal strains were caried out by the settle plate mediums and serial dilution method using Potato dextrose Agar (PDA) and malt extract Agar (MEA) mediums. A total of nineteen fungal strains (SA1 to SA19) including previously existing and seven isolated fungal strains were screened for polyethylene degradation by using pure polyethylene powders, beads, and films. Out of nineteen strains eleven showed the potentiality to degrade polyethylene powder which were further experimented with polyethylene beads. Ten fungal strains equally exhibited their potential to degrade polyethylene beads. These strains were further experimented with untreated, Ultraviolet (UV) rays (3 & 9 hours), and heat (45°C & 70°C) treatment. Five potential strains selected were further screened with 60°C heat followed by nitric acid and sodium hydroxide-treatment. Out of five, only two strains exhibited their capability to degrade polyethylene, hence plastic degrading enzymes were evaluated for these two strains and a prerequisite of 60°C heat followed by nitric acid treatment was selected for further experimentation.

Fungal enzymes laccase, Manganese peroxidase (MnP), protease, esterase, and lipase were qualitatively evaluated. Both strains showed the presence of these enzyme activities, but, only one strain with a prominent enzyme activity was selected for quantitative analysis. These enzyme activities were quantified and optimized for the selected fungal strain. Biotic (fungal consortia) and abiotic (chemical enhancers- Tween80, mineral oil, starch) augmentation of polyethylene degradation

was conducted by the selected fungal strain in soil and mulch medium at optimized conditions. In the end, a practical assessment of the polyethylene degradation method was conducted by supplementing the best enhancer in soil and mulch medium under simulated open field conditions. A model representing the actual processing method which could be practiced for a pollution free bioremediation of polyethylene has been also proposed which can be practically applied on field.

- **Study Area** includes temporary active dumping sites of different zones in Vadodara discrict of Gujarat i.e. (i) near to VUDA Bhawan (Karelibaug) for North zone, (ii) for east zone it is located near to Gadheda market (Waghodia), (iii) Atladara area for West & South zone; landfill area located at Jambuva area. Waste samples were collected from these sites for further experimentations.

- **Analysis of physical characteristics:** It revealed that approximately 80% of wastes have either the ability to be recycled or to be degraded naturally in the environment, the remaining 20% is causing damage to our ecosystems while ingested by mammals and is also considered to be non-biodegradable. Wastes like polyethylene bags and plastic food wrappers were highest in percentage in ten-year-old waste samples, thus such waste is required to be managed scientifically. Solid waste samples collected from the Jambuva landfill area showed different compositions in each depth. Solid waste samples from 0 ft. had the highest content of soil & unclassified debris and the lowest in 20 ft. waste sample which explains the decomposition of degradable waste. Kitchen waste was only found in 20 ft. waste samples i.e., 24.44% of total waste samples. Textile waste was observed in all three depth samples. Plastic waste includes different kinds of plastic products such as food wrappers, plastic bags of milk & other products, soap bottles & cosmetic packaging waste, disposable utensils, and polyethylene bags.

- **Isolation of fungal strains:** A collective number of seven fungal strains were isolated from air, soil, and leachate samples collected from MSW dumping sites. Fungal strains were identified based on morphological and microscopic characteristics. Three different species of *Aspergillus* sp. were identified based on its fast-growing fungal colonies with blue-green, green, brown, and black color and the notable structure of present phialides. A species of *Trichoderma* was isolated from soil and air; and later identified with its effuse colonies and conidiophores with lateral branches. Hyaline aerial hyphae, stolons, pigmented rhizoids, and differentiation into stolons & nodes with rhizoids were the main observed characteristics of *Rhizopus* species.

- **Screening of fungal strains (Powder, beads, films):** Polyethylene powder containing Czapek's dox media plates was inoculated with all nineteen fungal isolates to screen their degradation activity. A

cleared zone just below the fungal colony growth depicts the degradation of polyethylene powder particles by respective fungal species. In this experiment, among six strains of *Aspergillus*, four strains including two strains of *A. oryzae* (SA5 & SA15), *A. fumigates* (SA3) and *A. tubingensis* (SA1) showed positive results in degradation of HDPE & LDPE powders after three weeks. *A. oryzae* (SA5) fungal strain showed zone of clearance also in plates supplemented with LLDPE powder. Prominent clearance zones around the fungal colonies were seen in culture plates inoculated with *A. oryzae* (SA5 & SA15), *A. tubingensis*, and *Fusarium solani* MN201580.1 (SA17), out of nineteen strains eleven fungal isolates showed their inefficacy to break down polyethylene structure.

Although a few strains were not able to degrade powder particles supplemented with solid media, such strains gave 1-4% weight loss in LDPE beads after eight weeks of incubation. The highest weight-loss (7.70%) was witnessed in LDPE beads experimented with *A. oryzae* SA5 strain. Despite same fungal species, both the strains of *A. oryzae* (SA5 & SA15) were found to be showing differences in their capability to degrade polyethylene material. Strain SA15 took two months to degrade 5.05% of the bead, while strain SA5 was able to reduce polyethylene bead weight in fourteen days. *A. tubingensis*, *A. oryzae* (SA5 & SA15) were able to degrade 6.75%, 7.70% & 5.05% of LDPE beads, while *F. solani* MN201580.1 showed 6.92% of degradation in eight weeks at room temperature. This preliminary experiment concludes the potentiality of these three strains to degrade polyethylene material.

Ten species showed their potential to degrade polyethylene those are *A. tubingensis* (SA1), *A. oryzae* (SA5 & SA15), *A. fumigatus* (SA3), *T. viride* (SA7), *Trichoderma* sp. (SA8), *Rhizopus* sp. (SA12), *Pestalotiopsis* sp (SA13)., *F. solani* MN201580.1 (SA17), and *Flavodon* sp (SA19). These strains were selected for further degradation experiments with polyethylene films which are commonly found as plastic waste in MSW. Polyethylene (PE) films were pre-treated with Ultraviolet (UV) rays (3 & 9 hours) and heat (45°C & 70°C); untreated films were also kept along with pre-treated films. Weight-loss results revealed positive simulation in the degradation process by heat treatment as promising weight-loss percentages were observed in 70°C heat treated films. This experiment demonstrates the impact of heat treatment on degradation, as 5% to 47% weight-loss was observed in 70°C treated samples.

A. oryzae (SA15) presented highest weight loss 3.67% & 3.34% in 9 hours UV treated films after 4weeks & 6weeks of incubation. *A. oryzae* SA5 & SA15, *Pestalotiopsis* sp. and *F. solani* MN201580.1 gave percentage weight loss ranging from 2-3% in 3 hours UV treated films. While comparing untreated films and UV exposed PE films, films which were exposed to UV rays showed high amount of degradation than untreated films.

Samples were treated with 60°C heat followed by nitric acid and sodium hydroxide treatment. (I) 60°C heat + Concentrated HNo_3; (II) 60°C heat + 0.5M HNo_3; (III) 60°C heat + 0.5M NaOH These treated samples were experimented in mixture of soil + mulch media. This experiment revealed potentiality of *F. solani* MN201580.1 to degrade 21.33% of 60°C heat+ concentrated HNo_3 treated polyethylene film in two weeks without any sole source of carbon. In comparison with control (no treatment) treatment, weight-loss percentage had been increased 1-1.5% & 11-13% in heat & nitric acid treatment respectively. Increase in weight of all treated PE films experimented with both fungi was observed in later stages of incubation, depicting the formation of fungal biofilm on PE surface. Fourier transform infrared (FTIR) spectroscopy and Environmental- Scanning electron microscopic (E-SEM) analysis was performed on the experimented LDPE beads and PE films to confirm the degradation. In comparison to control polyethylene film (without fungal treatment) and A. oryzae 1 treated film, cracks and scratches had been witnessed on the surface formed due to fungal hyphae penetration. FTIR spectrum of heat-treated PE film experimented with *F. solani* MN201580.1 displays reduced peak intensity at 2920 cm-1 representing C-H stress. Increased peaks were observed at 1630 cm-1 and 1367 cm-1 wavenumber explaining production of carboxylic group. Carbonyl index of LDPE beads was found to be decreased in fungal treated sample compared to control samples (untreated). Previous reports have also proved decrease in the amount of carbonyl groups with prolonged exposure to a biotic environment (Dolezel, 1967).

- **Selection of Fungal species:** Although some species gave positive results in polyethylene powder and beads screening experiments, *A. oryzae* (SA5 & SA15) and *F. solani* MN201580.1 (SA17) seemed to be more potential in degrading almost all kinds of polyethylene components. Molecular identification was conducted for SA5, SA15, and SA17 strains. on basis of its DNA sequence, SA5 and SA15 strains were identified as *Aspergillus oryzae*, while SA17 was identified as *Fusarium solani* MN201580.1. On the basis of potentiality of SA15 and SA17 fungi, these strains were selected for the evaluation of enzymes.

- **Evaluation of polyethylene degrading enzymes:** Protease, lipase, esterase, laccase & manganese peroxidase (MnP) were qualitatively evaluated for selected fungal isolates *A. oryzae* SA15 and *F. solani* MN201580.1 by performing plate assay methods. Observations revealed that *F. solani* MN201580.1 & *A. oryzae* SA15 showed all responsible enzymes activity within one week of incubation period. In comparison of both the fungi, *F. solani* MN201580.1 inoculated plates showed prominent dark colored pigmentation around the colonies depicting the presence of ligninolytic enzymes. Lipase activity was confirmed by observing fluorescent orange-colored colonies

demonstrating the presence of lipase enzyme. Zone of hydrolysis for esterase and ligninolytic enzymes was prominent in *F. solani* MN201580.1 culture replicate, while production of lipase enzyme seemed to be faster in *A. oryzae* SA15 culture plate. Both the fungal species proved to be true producer of proteolytic, lipolytic and ligninolytic enzymes by developing hydrolysis zone. However, *F. solani* MN201580.1 strain had proved to be more potential polyethylene degrader processing the it all showed ability to produce all five polyethylene degrading enzymes, thus the fungus was taken for further research investigation.

Enzyme quantification in *F. solani* MN201580.1 was conducted by using different substrates. Different growth media (Malt extract, Casein & mineral salt media) were prepared for respective enzymatic activities. The medium was inoculated with single 9mm disc of 10 days old culture under aseptic condition and incubated at room temperature (25 ºC ± 1 ºC) for the desired incubation period. After respective incubation period fungal mycelium in the flask were homogenized by laboratory hand blender and filtered through pre-weighed Whatman paper no. 1 to collect the culture filtrate and fungal biomass.

Mycelial growth was noted as maximum (0.81gm) in malt extract medium than other two media. Laccase (2.25 U/ml) and protease (342.5 U/ml) enzyme activities were found highest on 25th day, maximum lipase (49.02 U/ml) & esterase (587.47 U/ml) were produced on 20th day whereas Mnp (0.2 U/ml) enzyme was highest on 15th day of incubation. Among all five enzymes, esterase & protease enzyme production was high in amount which has been reported as responsible polyethylene degrading enzymes. Thus, these quantification results revealed capability of *F. solani* MN201580.1 (SA17) to produce all five enzymes which are responsible for the break-down of polyethylene material.

- **Characterization and optimization of fungal enzymes** was performed to understand the effect of inoculum size, incubation time & temperature and pH level of the culture media on degradation process by *F. solani* MN201580.1 species.

Inoculum size and Incubation time

To check the effect of fungal inoculum size on the enzyme production, the experiment was executed with single disc and three discs of inoculum kept for nine incubation period starting from 3days to 35 days. In all five enzymes, single disc replicates produced more amount of enzymes than three discs inoculated culture replicates.

Highest production of laccase (1.58 U/ml), protease (314.5 U/ml) & Mnp (0.19 U/ml) enzymes in three discs inoculated culture replicates was noted on 20th day, while highest esterase (561.6 U/ml) &

lipase (48.71 U/ml) production was observed on 15th day of inoculation. Although single disc inoculated culture replicates produced enzymes slowly, had released more amount of enzymes than three discs inoculated replicates. Therefore, inoculum size was decided as single disc for further enzyme optimization experiments.

pH level

Fungal enzymes were assessed for their pH stability and enzymes were found to be stable at pH ranging from 4 to 8. Therefore, fungal growth and its capability to produce these enzymes at different pH level ranging from 4-14 pH of culture media was examined. In this experiment set-up, significant results were observed and fungal growth was seen highest at 8 pH culture media.

Temperature

Enzyme extracts were incubated at different temperature to check their stability and they found to be stable at temperature ranging from 10º C to 40º C. Production of these enzymes at different temperature ranging from 25º C to 45º C was examined by incubating culture replicates for desired period of time was also studied. Significant enzyme activity was observed at 30º C temperature and fungal biomass & enzyme activity found to be reduced with the high temperature.

Optimization of enzyme extraction methodology for solid state medium

All five enzymes were determined in medium containing soil and mulch by inoculating *F. solani* MN201580.1 and optimal parameters were maintained for the growth. To prepare the culture replicates, 50 gm of garden soil and 50 gm of mulch sterilized at 121º C for 30 minute and aseptically moistened with 50ml of sterile distilled water to create 60% moisture level. To standardize the suitable methodology for extracting all fungal enzymes, two different extraction methods were performed. Distilled water and buffer extraction methodology were tested and buffer extraction method detected optimum amount of enzyme activity.

- **Enhancement of polyethylene degradation:** It was experimented by supplementing chemical enhancers to the culture replicate and consortium technique for biological enhancement.

  A. Identification of potential consortium for biotic augmentation:

  Compatibility test: A paired interaction test was performed to check compatibility of all five potential fungal strains with each other. A 9 mm inoculum discs of two different strains were inoculated in a petri-plate and at different time intervals fungal growth was observed. Here in all ten paired interactions no interaction seemed to be having mutual intermingling instead they displayed partially intermingling phenomena which occurs when both fungi grow equally

without killing each other. *A. tubingensis* (SA1) and *F. solani* MN201580.1 (SA17) exhibited their compatibility with both the strains of *A. oryzae* (SA5 & SA17).

Screening of potential consortia: Ten co-culture and three consortium combinations of five potential fungal strains were experimented with untreated and heat treated (45°C & 70°C) PE films. Although the strains are compatible to each other, percentage weight loss results of these culture replicates had been found to be similar to their monoculture replicates indicating that no enhancement in enzyme activity took place. The combination of *A. oryzae* (SA15) and *F. solani* MN201580.1 (SA17) fungal strains did not seem to be beneficial for the increased weight-loss percentage. Non-compatible partners also failed to exhibit reduction (0%) in PE film. On comparison of single culture technique gave 21.33% weight-loss which is higher than co-culture technique. Hence this experiment had proved the potentiality of *F. solani* MN201580.1 to degrade polyethylene as single culture.

B. Addition of chemical enhancers for abiotic augmentation: The degradation activity could be increased with shorter period of time by adding enhancers such as 0.05% mineral oil, Tween 80 and soluble starch to the media. Previously mentioned thermo-chemical pretreatments were performed on PE films and fungal strains were inoculated along with chemical enhancers and kept for desired incubation periods (2, 4, 6, 8, 12 & 16 weeks). Similar to earlier results 60° C heat treatment followed by concentrated nitric acid treatment proved to be effective for the degradation process.

Among all three chemical enhancers Tween 80 provided highest weight reduction (38.8%) in 60° heat + concentrated HNo_3 treated PE films after fourteen days. Starch and mineral oil also induced the degradation process by stimulating fungal growth on polyethylene film. Maximum weight reduction in starch supplemented culture replicates was 15.2% after two weeks of incubation, while in mineral oil added replicates the maximum reduction was 12.47%. However, the weight of films gradually increased due to fungal growth on the surface and eventually films tested for longer time period showed increase in weight. Topographic changes like black and brown patches, small holes on the surface, wrinkled PE samples and mycelial growth were observed on films experimented with all three inducers.

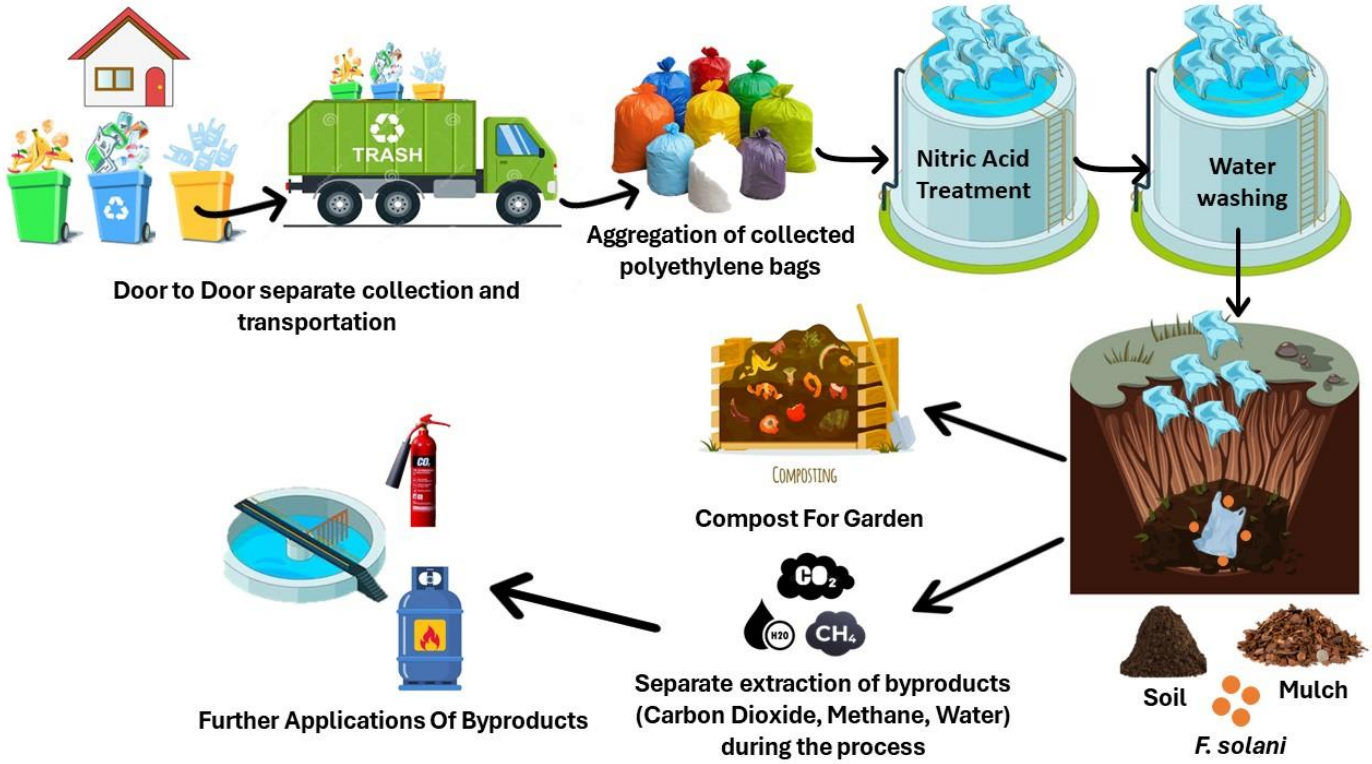
❖ **On field assessment of polyethylene degradation:** The experiment was conducted in two parts; the protocol was experimented with in a lab-simulated area and followed by experimentation in an open-field area. The experiment was set up in earthen pots which were filled with soil & mulch. The films were experimented with using *F. solani* MN201580.1 (SA17) as the degrading agent within a

composite medium of soil and mulch, soil+mulch media supplemented with tween 80 while maintaining optimal conditions. The degradation was confirmed by weight-loss analysis, evaluation of enzymatic activity, Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) analysis. Additionally, advanced analytical techniques, namely Differential Scanning Calorimetry (DSC) and Gel Permeation Chromatography (GPC), were carried out for the comprehensive assessment of the modified polyethylene films. In this set-up, *F. solani* MN201580.1 (SA17) was identified as a potential degrader of polyethylene film, as maximum weight loss was recorded after 20 days with 40 ± 0.14% and 41.5 ± 0.56% in lab-stimulated and open-field experiments, respectively. Further analysis unveiled changes in topography, chemical composition, thermal behavior, molecular weight, and tensile strength of the experimented film.

To summarize, the present research work demonstrates that *Fusarium solani* MN201580.1 (SA17) has a remarkable capacity to break down the polymeric chain of pre-treated polyethylene film when experimented in soil and mulch medium with tween 80 supplementation. The present research study has established a scientific and biological solution to existing plastic waste, offering practical implications for sustainable waste management.

Recommended model plan for polyethylene degradation

The comprehensive model plan for polyethylene degradation includes the segregation of polyethylene waste at home and transportation to the landfill. The subsequent treatment is to immerse the waste into concentrated nitric acid in a large tank with precise safety measures and washing it with water. Further, the waste is to be placed in soil and mulch along with *Fusarium solani* MN201580.1 and tween 80. The process employs separation and capturing of by-products methane (CH_4), water (H_2O), and carbon dioxide (CO_2) through efficient separators. The methane gas is designated for utilization as a clean fuel in sanitization and automobiles. The collected water undergoes a de-pollution process via wastewater treatment, ensuring its suitability for reuse. Carbon dioxide, once collected, is transformed into a powder form for use in refrigerators and fire extinguishers. Additionally, the degraded soil and mulch are repurposed as nutrient-rich compost for gardens. This protocol can be considered as a holistic approach to sustainable waste management.



Protocol model explaining the field set up for polyethylene degradation on landfill