Synopsis To The Thesis

Municipal Solid Waste Characterization And Its Assessment For Fungal Bioremediation

Submitted to

The Maharaja Sayajirao University of Baroda Vadodara

Submitted by

Ms. Mitu Mewada

Guided by

Prof. Susy Albert
Department of Botany
Faculty of Science
The Maharaja Sayajirao University of Baroda Vadodara

Growing rate of population influences 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 of countless small components known as monomers. The widespread use of plastics during past decade in all the sectors of life has generated serious problems with plastic waste due to its accumulation in the environment (Sowmya et al., 2015). 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 great tensile strength than LDPE (Satlewal et al., 2008). While LLDPE also has higher tensile strength and higher impact and puncture resistance than LDPE material.

All kinds of plastics have immensely been used during past decade in all production sectors resulting into serious problems due to its 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).

Prior to the COVID-19 episode, several countries were considering about prohibition on single-use plastic items as a war was being pursued against single-use plastics. This pandemic scenario has raged the plastic threat in forms of PPE coveralls, face shields, gloves & masks, body bags, packaging of sanitizer dispensers and restaurant takeaways.

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 breakdown 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 are given by Kale et al.,(2015) in which it can be understood that there are a large number of potential fungal strains which have the capacity to degrade plastics.

Though there are lots of reports demonstrating the potential of plastic degrading microbes, but none of them found to have practical application, thus there is a strong need to screen efficient organisms and developing technologies capable of degrading plastic efficiently without affecting the environment. Their main feature in bioremediation mechanism is the production of extracellular enzymes like laccases, peroxidases & esterases and are directly initiating microbial attachment on the PE surface and the consequent biodegradation (Wei and Zimmermann 2017). However, there are fewer reports available involving enzymes responsible the PE degradation process.

This research aspect requires in-depth study of the enzymes involved in this process, enhancement of the degradation and its potentiality under natural conditions. The present study emphases on

finding out the non-biodegradable component of MSW and identifying a potential fungal strain for the degradation of non-biodegradable element especially the plastics.

OBJECTIVES

- Compositional analysis of municipal solid waste (MSW) of Vadodara city.
- Identify fungal diversity of municipal solid waste
- Screening, identification and selection of potential plastic degrading fungi.
- Evaluation of plastic degrading fungal enzymes
- Characterization and optimization of enzyme degradation by the selected fungi
- Enhancement of plastic degradation under optimized condition using different enhancers. (Chemical & Biological)
- Practical experiment and assessment of fungal biodegradation under natural conditions.

SUMMARY OF RESULTS

- **Study Area** includes temporary active dumping sites for different zones; (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 site for further experimentations.
- **Compositional analysis** of Municipal solid waste (MSW) from different dumping sites in Vadodara was conducted. The main landfill area is located at Jambuva area with 750 TPT waste. Daily waste is being dumped at temporary; where segregation takes place and remaining waste is

transferred to the main landfill area. Waste samples were collected from all the three temporary dumpsites and Jambuva landfill area for the study of physical characteristics of MSW.

Analysis of physical characteristics revealed that approximately 80% of wastes have either ability to be recycled or to be degraded naturally in the environment, rest 20% is causing damage to our ecosystems while ingested by mammals and also considered to be non-biodegradable. Wastes like polyethylene bags and plastic food wrappers were highest in percentage in ten-years old waste samples, thus such waste is required to be managed scientifically.

Solid waste samples collected from Jambuva landfill area showed different composition in each depths. Solid waste sample from 0 ft. had highest content of soil & unclassified debris and lowest in 20 ft. waste sample that explains decomposition of degradable waste. Kitchen waste was only found in 20 ft. waste samples i.e. 24.44% of total waste sample. Textile waste was observed in all three depths samples. Plastic waste includes different kind 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** was executed by performing settle plate method and serial dilution on Potato dextrose Agar (PDA) & Malt extract Agar (MEA). A collective number of seven fungal strains were isolated from air, soil and leachate samples collected from MSW dumping sites.

  Fungal strains were identified on the basis of morphological and microscopic characteristics. Three different species of *Aspergillus sp.* were identified on the basis of its fast growing fungal colonies with blue-green, green, brown and black color and the notable structure of present phialides (Nagmani A. et al., 2016).

A species of *Trichoderma* was isolated from soil and air; 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. *A. oryzae* strain was sent for confirmation of its identity by DNA sequencing which was performed by first Base DNA sequencing Services, Malaysia**.** Molecular identification was conducted for SA15 strain, on basis of its DNA sequence the strain was identified as *Aspergillus oryzae*.

- **Screening of fungal strains** for polyethylene degradation was conducted by using polyethylene powders, beads and films. Polyethylene powder containing Czapek's dox media plates were inoculated with all nineteen fungal isolates to screen their degradation activity. A cleared zone just below the fungal colony growth depicts 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*, *A. fumigates* and *A. tubingensis* 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 roseum*, out of nineteen strains eleven fungal isolates showed their inefficacy to break-down polyethylene structure.

Although few strains had not able to degrade powder particles supplemented solid media, such strains gave 1-4% weight-loss in LDPE beads after eight weeks of incubation. Highest weight-loss (7.70%) was witnessed in LDPE beads experimented with *A. oryzae* SA5 strain. In spite of same fungal species, both the strains of *A. oryzae* (SA5 & 15) were found to be showing difference in their capability to degrade polyethylene material. Strain SA15 took two months to degrade 5.05%

of bead, while strain SA5 could 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. roseum* showed 6.92% of degradation in eight weeks at room temperature. This preliminary experiment concludes potentiality of these three strains to degrade polyethylene material.

Ten species showed their potential to degrade polyethylene those are *A. tubingensis, A. oryzae* SA5 & 15, *A. fumigatus, T. viride*, *Trichoderma* sp., *Rhizopus* sp., *Pestalotiopsis* sp., *F. roseum,* and *Flavodon* sp. 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 degradation process by heat treatment as promising weight-loss percentage were observed in 70°C heat treated films. This experiment clearly demonstrates the impact of heat treatment on degradation, as 5% to 47% weight-loss were 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. roseum* 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.

Fourier transform infrared (FTIR) spectroscopy and Environmental- Scanning electron microscopic (E-SEM) analysis were 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. roseum 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 & 15 and *F. roseum* seemed to be more potential in degrading almost all kinds of polyethylene components. Therefore, these two fungal strains were selected for further evaluation experiments.

- **Polyethylene degrading enzymes** such as protease, lipase, esterase, laccase & manganese peroxidase (MnP) were qualitatively evaluated for selected fungal isolates *A. oryzae* SA15 and *F. roseum* by performing plate assay methods. Culture plates were observed for one week to observe the fungal growth and the clear zone below fungal colonies. Observations revealed that *F. roseum* & *A. oryzae* SA15 showed all responsible enzymes activity within one week of incubation period. In comparison of both the fungi, *F. roseum* 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. roseum* 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. roseum* strain had proved to be more potential polyethylene degrader fungal species and it all showed ability to produce all five polyethylene degrading enzymes, thus the fungus was taken for further research investigation.

Enzyme quantification in *F. roseum* 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 blander 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. roseum* 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. roseum* 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 20^{th} day, while highest esterase (561.6 U/ml) & lipase (48.71 U/ml) production was observed on 15^{th} 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.

- **Enhancement of polyethylene degradation** was experimented by pre-treating films, adding chemical enhancers to the culture replicate and also consortium technique for biological enhancement.

  A. **Thermo-chemical treatment to the polyethylene film for chemical enhancement:** 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.

  In this experiment *A. oryzae* SA15 showed 15.42% weight reduction in 60°C heat+ concentrated HNo_3 treated PE film in two weeks of incubation. While this experiment revealed potentiality of *F. roseum* 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.

B. **Identification of potential consortium for biological enhancement:**

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* and *F. roseum* exhibited their compatibility with both the strains of A. oryzae. As shown in Figure.8. in paired interaction of *F. roseum* with *Pestalotiopsis* sp. the growth of fungi seemed to be stopped once they came in contact with each other and over growth of *A. tubingensis* killed *Pestalotiopsis* sp. Screening of potential consortia: Compatible strains were grown together as consortium to check the enhanced degradation activity. Below mentioned three various consortiums of five potential fungal strains were experimented with untreated and heat treated (45°C & 70°C) PE films.

1) *A. oryzae* (SA5) + *A. oryzae* (SA15) + *F. roseum* (SA17);

2) *A. oryzae* (SA5) + *A. oryzae* (SA15) + *F. roseum* (SA17) + *A. tubingensis* (SA1);

3) *A. oryzae* (SA15) + *F. roseum* (SA17).

Although these strains are compatible to each other, percentage weight loss results of these culture replicates had been found to be similar to their monoculture replicates. The combination of *A. oryzae* and *F. roseum* fungal strains did not seem to be beneficial for the increased weight-loss percentage. In comparison of single culture and co-culture experiment, single culture technique gave 21.33% weight-loss which is higher than co-

culture technique. Hence this experiment had proved the potentiality of *F. roseum* to degrade polyethylene as single culture.

C. **Addition of chemical enhancers:** 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.

REMAINING RESEARCH WORK

- FTIR and SEM analysis of fungal treated PE films will be conducted to confirm the degradation occurrence by *F. roseum* species.
- Final assessment of polyethylene biodegradation will be examined under optimized conditions by supplementing enhancers to natural culture media (Soil + Mulch).

Date:

Research guide
Prof. Susy Albert

Head
Department of Botany

Dean
Faculty of Science

Encl: Details of publications and paper presentations

PUBLICATIONS

Sr. No.	Title of paper	Authors	Name & Vol. of Journal & Year	Details
1.	Municipal solid waste management system in Vadodara city: Current scenario	**Mewada, M.,** Albert, S. and Padhiar, A.	IOSR Journal of Environmental Science, Toxicology and Food Technology	14 (2020), 45-50
2.	Screening of fungal microbiome to identify potential polyethylene degrading fungi	**Mewada, M.,** Albert, S., Taunk, A. and Bhatt, K.	Journal of Solid Waste and Technology and Management	47|4 (2021), 619-626
Paper Accepted				
1.	Long-term environmental impact of COVID-19 pandemic: Derailed single-use plastic ban	**Mewada, M.,** Albert, S.	International journal of Environment and waste management	
Other Publications				
1.	A comparative account of micromorphological and anatomical studies on mangrove species of Gujarat	**Mewada, M.,** Albert, S., Pathak, P. and Dodiya, J.,	Indian Biodiversity Congress- 2014, Perspectives on Biodiversity of India	Vol. II. Part-1 (2016), 192-198
2.	Enhancement of ligninolytic & xylanolytic enzyme activities in Trichoderma reesei co-cultured with two white rot fungi	**Mewada, M.,** Albert, S. and Pandya, B.	International Journal of Biotechnology and Biochemistry	13|4 (2017), 429- 439
3.	Evaluation of potential compatible co-partner for lignin degrader Irpex lacteus	**Mewada, M.,** Pandya, B. and Albert, S.	International Journal of Experimental Biology	56 (2018), 764-768

PAPER PRESENTATIONS

Sr. No.	Title of paper	Authors	Details	Themes and Dates
1.	Characterization and isolation of fungal strains from municipal solid waste	**Mewada, M.,** Albert, S. and Padhiar, A.	XLI All India Botanical Conference	National Symposium on Ecological Restoration, Carbon Sequestration & Biotechnological approaches for Biodiversity Conservation (25^{th}-27^{th} Oct, 2018), Jiwaji University, Gwalior.
2.	Screening of fungal microbiome from municipal solid waste dumping site to identify potential polyethylene degrading fungi	**Mewada, M.,** Albert, S. and Padhiar, A.	International Symposium by Mycological Society of India (MSI)	Fungal Biology: Advances, Application & Conservation (19^{th}-21^{st} Nov,2018), Agharkar Research Institute (ARI), Pune
3.	Mycoremediation for Municipal solid waste management	**Mewada, M.,** Albert, S. and Padhiar, A.	SOCLEEN Seminar	Green Home: Smart solutions (10^{th} Feb, 2019), Vadodara.
4.	Polyethylene fungal degradation: A solution to plastic menace	**Mewada, M.,** Albert, S. and Padhiar, A.	XLII All India Botanical Conference	National symposium on Innovations and inventions in plant science research (6^{th}-8^{th}, November, 2019), University of Calicut, kerala.
5.	Fungal degradation of Polyethylene: A biotechnological perspective to plastic pollution	**Mewada, M.,** Albert, S. and Padhiar, A.	International Conference by Navrachana University	1st International Conference on Ecohealth and Environmental Sustainability" (ICEES) in collaboration with the University of Calgary, Alberta, Canada, 24-26 February 2020, Navrachana University, Gujarat