CHAPTER 2

Screening for potential plastic degrading fungal species

Screening process was conducted to assess the degradation ability of previously isolated fungal strains, as well as additional strains that were procured from National Fungal Culture Collection of India (NFCCI). The screening was conducted on the raw material used as the main components of polyethylene bags, namely High-Density Polyethylene (HDPE), Low Density Polyethylene (LDPE), and Linear-Low Density Polyethylene (LLDPE) materials. These materials are commonly used in the production of plastic shopping bags.

This assessment typically involves laboratory studies and tests to assess the strain's ability to metabolize and break down hydrocarbons under controlled conditions. The experiment involved testing the fungal strains with HDPE, LDPE, and LLDPE powders and beads. The aim was to identify fungal species that showed potential for degrading these polyethylene materials.

The selected strains were then further tested using untreated and pretreated polyethylene films to evaluate their degradation capabilities. The screening of strains against polyethylene films was conducted in three stages. Initially, the strains were tested on photo-oxidized films. The species that exhibited the best results in this stage were selected for further screening using thermo-oxidized films. Finally, the most promising species were identified through screening with thermo-chemically oxidized films.

METHODOLOGY

Polyethylene materials

HDPE & LLDPE Powders were obtained from ONGC Petro additions Ltd., Dahej, Gujarat, while LDPE powder was prepared in the laboratory by boiling LDPE beads with xylene followed by solvent evaporation at room temperature. Prepared LDPE powder was successively washed with ethanol (Satlewal et al., 2008). LDPE beads were obtained from Reliance Pvt. Ltd., Jamnagar and Billion Flex Pvt. Ltd., Halol, Gujarat, India.

Polyethylene bags with 10 microns thickness were procured from the local shop and were cut into 1×1 cm pieces and weighed for the degradation experiments. The samples were weighed after pre-treatments and the weight was considered as initial weight for the weight loss analysis.

Screening of Polyethylene powder:

HDPE, LDPE & LLDPE powders (0.5g/l) were supplemented in Czapek's dox media with minimal nutrients (Maren, 2002) & fungal strains were inoculated for screening of degradation. A cleared zone just below the fungal colony growth depicts degradation of polyethylene powder particles by respective fungal species (Sowmya et al., 2015).

Screening of Polyethylene beads:

Fungal strains were inoculated on Czapek's dox media and after one week of fungal growth pre-weighed LDPE beads were aseptically inserted and kept for 4-, 6- & 8-week incubation periods at room temperature 25-30°C. After desired incubation period experimented beads were removed and washed with 70% ethanol to determine weight-loss. Percentage change in weight, was determined using the formula:

Change in weight/Initial weight × 100 = Percentage change in weight

Screening Polyethylene films:

-Preparation of broth culture replicates for the degradation

The pre-weighed and pre-treated films were aseptically transferred into 100 ml of Malt extract broth medium and kept for desired incubation periods (2, 4 & 6 weeks) after inoculating with 9mm disc of fungus. The tests were performed in triplicate for each fungal strain. A set of control experiments were performed containing only the PE films in growth medium devoid of fungal inoculum. After each incubation period, films were carefully removed and washed with 2% Sodium dodecyl sulphate (SDS) for 5 hours to remove fungal mycelia (Kyaw et al.,2012), washed thoroughly in distilled water and drained on filter paper to remove excess water & dried at room temperature for 8 hours.

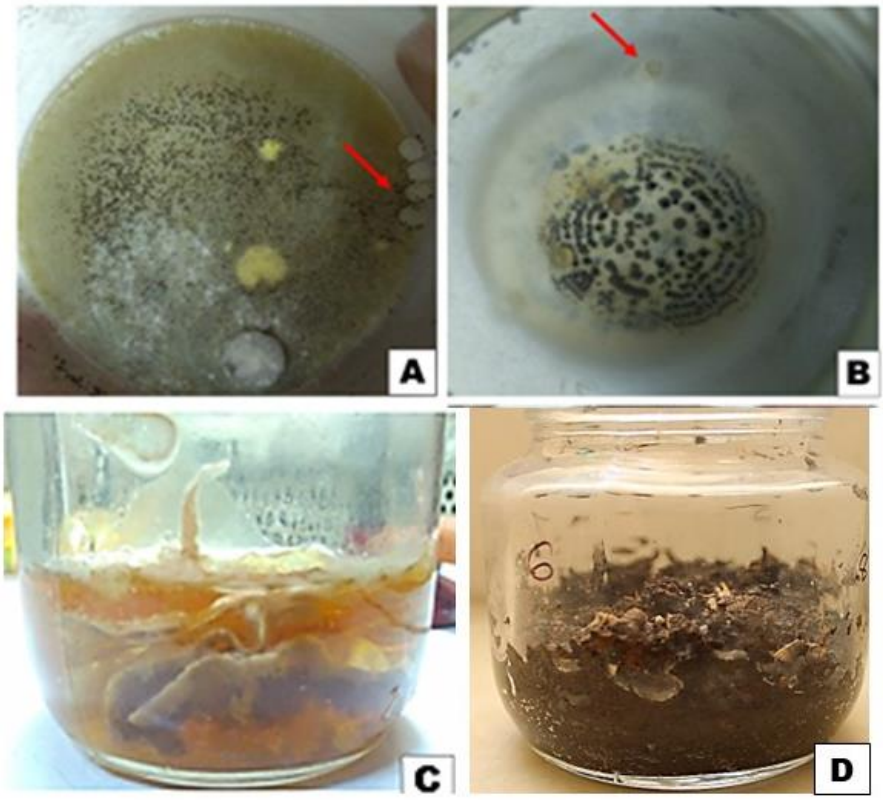

Figure.9. Experiment set up for screening of fungal strains with LDPE beads and Polyethylene bag films: A & B- Polyethylene bead covered under fungal mycelial and spores**; C & D-** Polyethylene films screened with fungal strains in Malt extract Broth and soil+mulch medium

-Pretreatment of the polyethylene film

Polyethylene films were pre-treated before inoculating it with the fungus. The treatment with best results was selected for the further experimentation. Following pretreatments were given to the film to enhance the fungal degradation:

(I) Photo-oxidation: Polyethylene films were photo-oxidized for the duration of 3 hours and 9 hours under a long-wave UV lamp (365 nm). (Ho and Pometto, 1999; Lee et al.,1991)

(II) Thermo-oxidation: Films were pre-treated with 45°C & 70°C heat by keeping uninterrupted in hot air oven for 24 hours. (Corti et al., 2010; Suresh et al., 2011)

(III) Thermo-chemical treatment: Samples were treated with 60°C heat followed by nitric acid and sodium hydroxide treatment. Treated samples were experimented in mixture of soil + mulch media (Meinel, G., & Peterlin, A. (1968; Nakae et al., 2000; Nwachukwu et al., 2010).

FTIR spectroscopy and SEM analysis

Experimented samples (Bead) were analyzed by FTIR (Fourier Transform Infrared) to determine different peaks relative to deformation, symmetrical & asymmetrical bending, stretching of CH_2, CH stretching and C-O bond were compared taking non-experimented HDPE/LDPE samples at Gujarat Industrial Research and Development Agency (GIRDA), Vadodara, Gujarat, India. Morphological changes on polyethylene beads were observed under E-SEM (Environmental- Scanning Electron Microscope) at Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Anand, Gujarat, India.

Carbonyl Index

Change in Carbonyl Index (CI) is also known to be a factor which proves degradation of Polyethylene; thus, CI was calculated from absorbance at 1710 and 1380 cm^{-1} wavenumber using the standard formula mentioned below. (Ojha et al., 2017)

Carbonyl Index= Absorbance at 1710 cm^{-1} (the maximum of carbonyl peak) /Absorbance at 1380 cm^{-1} (the maximum of carbonyl peak)

Statistical Analysis

Screening experiments were carried out in triplicates and the values are presented in mean value with standard deviation (Mean ± SD). A significance analysis was conducted on observed readings with two-factor ANOVA (Analysis of Variance) with α as 0.05.

RESULT & DISCUSSION

Screening of fungal strains on different polyethylene with different densities:

Microorganisms are accounted to be equipped for transforming plastic into their ecological niche and it could be termed as 'plastisphere' (Amaral-Zettler et al., 2020). Numerous fungal and bacterial species demonstrated their capacity in breaking down the refractory atom, including polymer, converting them into non-toxic composites. Albeit past investigations have reported bacteria-driven biodeterioration, fungal species are more potential creatures in the degradation of polyethylene (Muhonja et al., 2018).

Myco-organisms are strikingly versatile and their tendency continues to evolve and the characteristics of the fungi can change due to the existence of polymeric molecules (Esan et al., 2019; Wiedner and Polifka 2020). Anastasi et al. (2013) stated that the fungi organisms are capable to colonize on all kinds of mediums and survive in diverse environments, where they support the ecological equilibrium. Subsequently, bacterial isolates were excluded from the further screening examinations and different fungal isolates were screened with LDPE, LLDPE, and HDPE powders & beads.

The main difference among HDPE, LDPE, and LLDPE lies in their chemical structures and the presence of branching. HDPE (High-Density Polyethylene) has a relatively low amount of long-chain branching in its chemical structure. This results in a more linear and tightly packed molecular arrangement, leading to a higher density and stronger material.

On the other hand, LDPE (Low-Density Polyethylene) possesses a higher amount of long-chain branching compared to HDPE. This branching introduces irregularities in the molecular structure, reducing the density and creating a more flexible and less dense material.

LLDPE (Linear Low-Density Polyethylene) incorporates a significant number of short-chain branches within its chemical structure. These short-chain branches prevent the polymer chains from packing closely together, resulting in a material with improved flexibility, impact resistance, and tensile strength compared to LDPE. The variation in the degree and type of branching in HDPE, LDPE, and LLDPE contributes to their distinct properties and applications. Polyethylene bags are manufactured using different proportions of HDPE, LDPE, and LLDPE, depending on the desired properties and intended applications of the bags.

Producers can vary the ratios of HDPE, LDPE, and LLDPE to achieve specific characteristics in the final product. For example, bags that require higher strength and rigidity may have a higher proportion of HDPE. Bags that need to be more flexible and resistant to impact may have a higher proportion of LDPE or LLDPE.

Polyethylene Powders:

Figure.10-12 depict the results obtained of screening different fungal strains after four weeks of inoculation on three polyethylene powders supplemented with solid media. Similar research studies reported that polyethylene serves as a sole carbon source for microbial growth (Tiso et al., 2022; Niu et al., 2023; Alshehrei, 2017). 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.

Different species of *Aspergillus* have been reported for its ability for the breakage of polymeric bonds. *Aspergillus nidulans* and *A. flavus* isolated from garbage soil samples, reported for their potentiality to degrade LDPE powder (Usha et al., 2011). Fatimah (2017) observed the efficacy of *A. niger*, *A. flavus*, *A. terreus* and *A. fumigatus*, the colony growth on medium supplemented with LDPE powder appeared to be prominent. In another study, *A. flavus* and *A. tubingensis* showed capability to breakdown two commercially used polymers polyurethane (PU) & Polyethylene by releasing the extracellular enzymes utilizing the polymer as carbon base (Devi et al., 2015; Mathur & Prasad, 2012; Khan et al., 2017).

Table.7. Screening of fungal strains with Polyethylene powders

Sr. No.	Fungal species	Polyethylene Powders		
		HDPE	LDPE	LLDPE
1	*Aspergillus tubingensis*	√	√	-
2	*Aspergillus flavus*	-	-	-
3	*Aspergillus fumigatus*	√	√	-
4	*Aspergillus niger*	-	-	-
5	*Aspergillus oryzae* SA5	√	√	√
6	*Trichoderma reesei*	-	-	-
7	*Trichoderma viride*	√	-	-
8	*Trichoderma* sp.	√	√	-
9	*Pennicillium oxalicum*	-	-	-
10	*Pennicillium chrysogenum*	-	-	-
11	*Pennicillium citrinum*	-	-	-
12	*Rhizopus* sp.	-	-	-
13	*Pestalotiopsis* sp.	√	-	-
14	*Curvularia* sp.	-	-	-
15	*Aspergillus oryzae* SA15	√	√	-
16	*Pleurotus sajorkaju*	-	-	-
17	*Fusarium solani*	√	√	√
18	*Phanerochaete chrysosporium*	-	-	-
19	*Flavodon* sp.	-	-	-

(Highlighted box represents prominent zone of clearance observed in polyethylene powders by respective fungal strain)

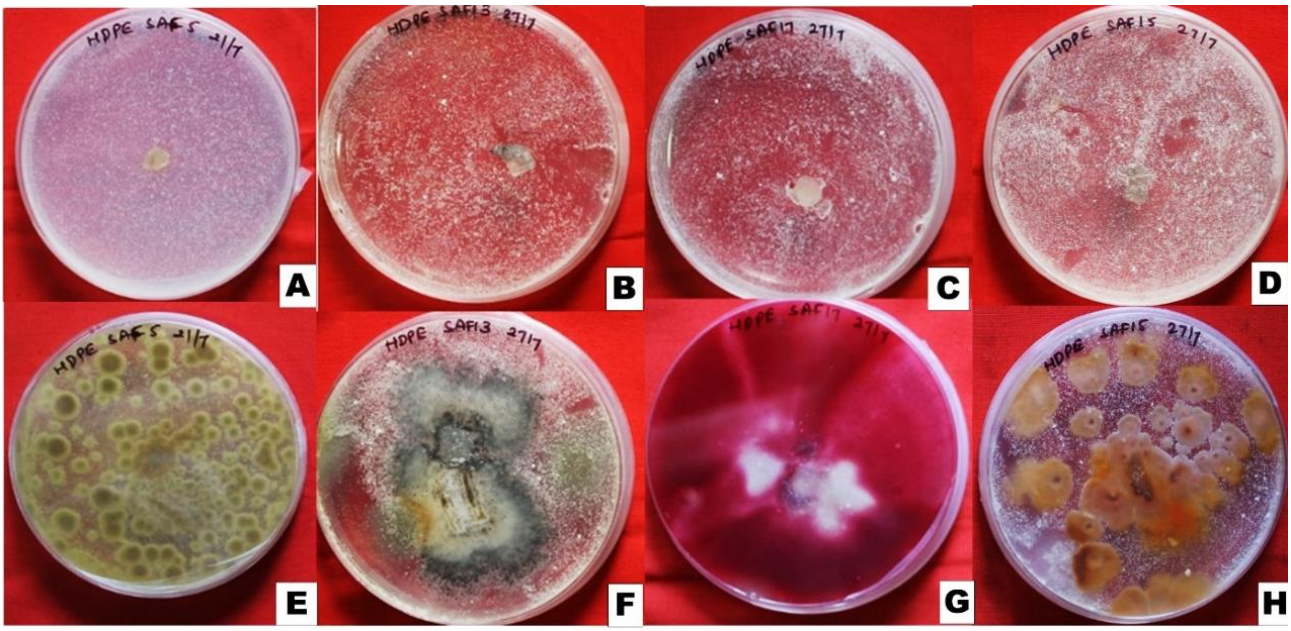

Figure.10. Screening of fungal strains with HDPE powder: (A-D) HDPE powder supplemented in solid media; (E-H) Zone of clearance appeared after four weeks of incubation period
A & E- *A. oryzae* SA5; **B & F**- *Pestalotiopsis* sp.; **C & G**- *F. solani*; **D & H**- *A. oryzae* SA15

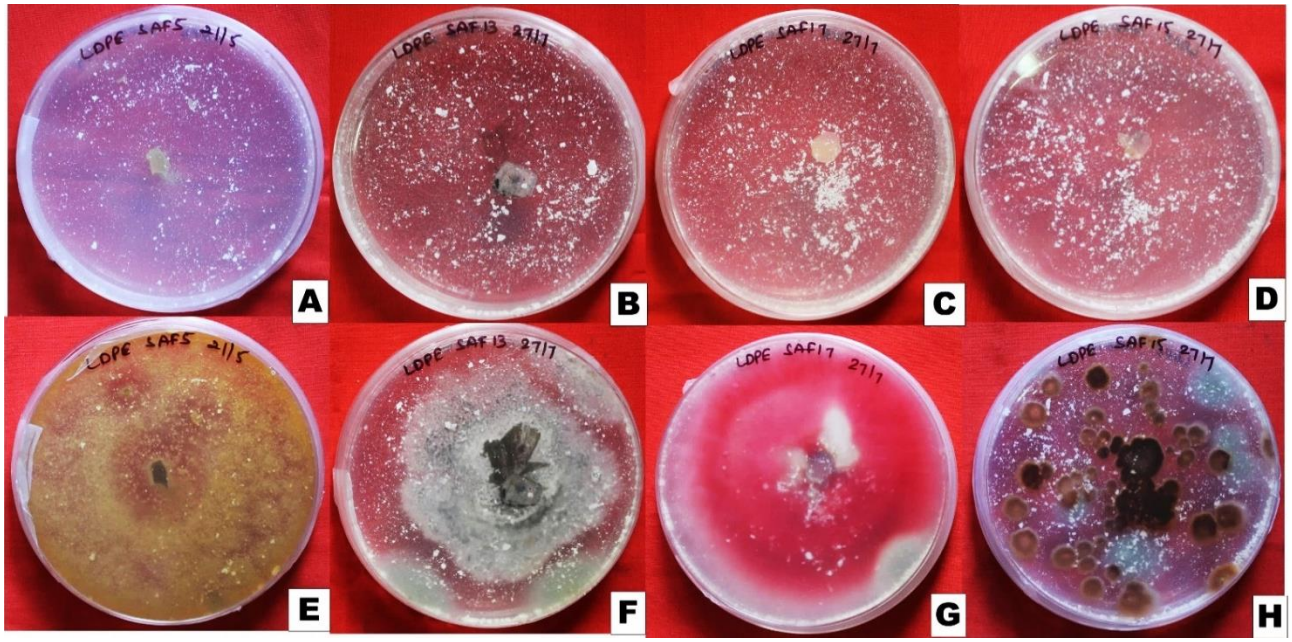

Figure.11. Screening of fungal strains with LDPE powder: (A-D) LDPE powder supplemented in solid media; (E-H) Zone of clearance appeared after four weeks of incubation period
A & E- *A. oryzae* SA5; **B & F**- *Pestalotiopsis* sp.; **C & G**- *F. solani*; **D & H**- *A. oryzae* SA15

Some strains of *Aspergillus* have been documented to have the ability to degrade hydrocarbons, including aromatic compounds and complex organic materials (Kumar et al., 2021; Das and Chandran, 2011; Asemoloye et al., 2020). In this experiment, among six strains of *Aspergillus*, four strains including two strains of *A. oryzae*, *A. fumigatus* and *A. tubingensis* showed positive results in degradation of HDPE & LDPE powders after three weeks. *A. oryzae* STR1 (SA5) fungal strain showed zone of clearance also in plates supplemented with LLDPE powder can be clearly observed

in Figure.12 (A & E). Many study reports have indicated the ability of *A. flavus* species to break down polymer structure (Saxena et al., 2022; Ma and Wong, 2013), however, in this investigation, *A. flavus* strain did not develop the clearance zone, which describes its inability to degrade all three types of polyethylene powders.

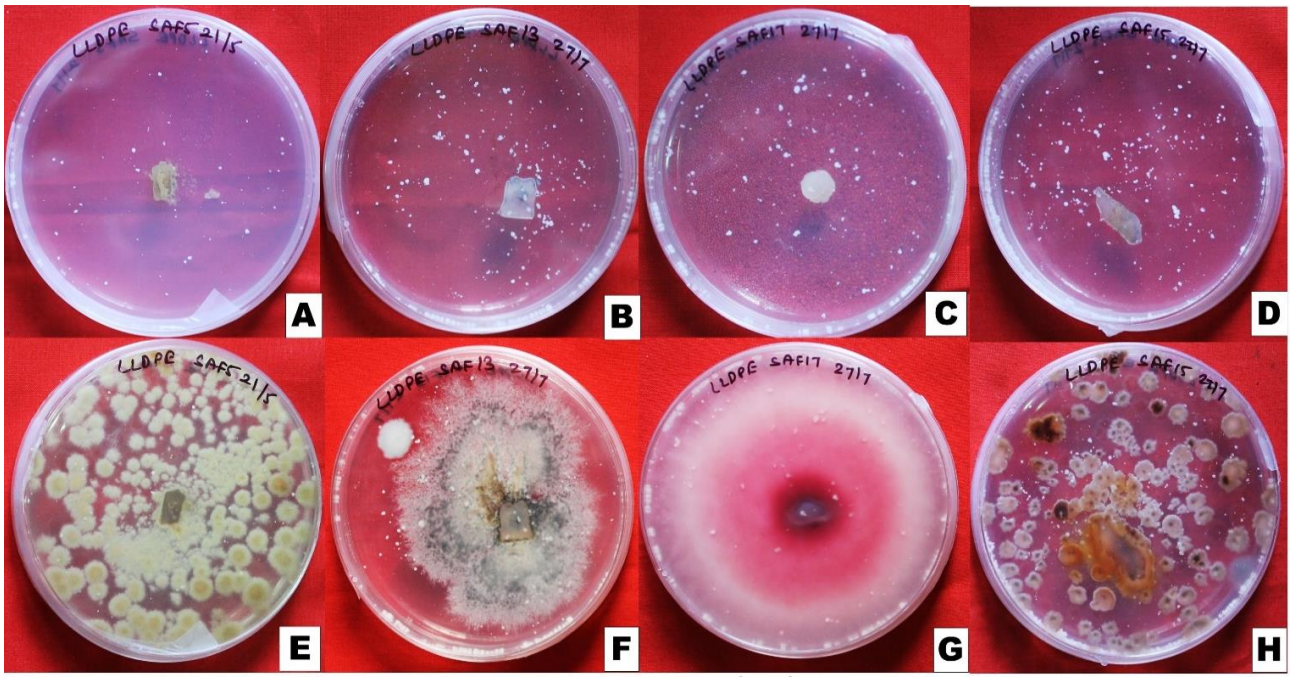

Figure.12. Screening of fungal strains with LLDPE powder: (A-D) LLDPE powder supplemented in solid media; (E-H) Zone of clearance appeared after four weeks of incubation period
A & E- *A. oryzae* SA5; B & F- *Pestalotiopsis* sp.; C & G- *F. solani*; D & H- *A. oryzae* SA15

The biodegradation of LDPE by *A. oryzae*, *A. fumigatus* & *A. niger* had been reported under in vitro conditions in the solid medium (Konduri et al., 2010; Zahra et al., 2010; Esmaeili et al. 2013). Unlike reported degradation activity of *A. flavus* and *A. niger*, these isolates did not demonstrate their potentiality to degrade polyethylene materials.

These differences in degradation capability can arise due to genetic variations and adaptations within a species. Environmental factors, such as exposure to specific pollutants over time, can also influence the selection and evolution of strains with enhanced degradation capabilities. This implies that the isolated strains of *Aspergillus* may have different metabolic capabilities or enzymatic profiles than those previously documented for hydrocarbon degradation.

Fusarium solani grew well on all the three different polyethylene powders and could break up the powder particles of HDPE, LDPE & LLDPE resulting in a clearance zone around the growing culture (Figure. 10-12, D&G). Raghavendra et al. (2016) found *Fusarium* and *Aspergillus* species as the most widely recognized indigenous fungi in plastic waste of landfills from different regions of India. *Fusarium oxysporium*, *F. solani* and other unknown species have been already associated with

polymer degradation (Das & Kumar, 2014; Taxeidis et al., 2023; Wróbel et al., 2023). Therefore, this strain of *F. solani* could exhibit significant degradation rate.

Research study driven by Singh et al. revealed LDPE degradation potentiality of *Penicillium* sp. with 6.58% degradation (Singh and Gupta 2014). In the present study identified *Penicillium* species did not show degradation. *Trichoderma harzianum* has been proven to be efficient degrader of UV-treated polyethylene using it as a sole source of carbon (Sowmya et al., 2014). In the present investigation, *Trichoderma viride* (SA7) and unknown species of *Trichoderma* sp. (SA8) had been observed to be capable of degrading polyethylene powder particles supplemented in solid media.

Pestalotiopsis microspora isolated from Ecuadorian rainforest, has been reported to have the ability to degrade polyurethane polymer which is usually used in manufacturing paints and adhesive (Russell et al., 2011), while in the present experiment HDPE powder particles were degraded by *Pestalotiopsis* species (SA13). Prominent clearance zones around the fungal colonies were seen in culture plates inoculated with *A. oryzae* SA5 (Figure.10- A&E) & SA15 (Figure.10- D&H), *A. tubingensis, and Fusarium solani* (Figure.10- C&G). Out of nineteen strains screened eleven fungal isolates showed their inefficiency to break-down polyethylene structure as there was no significant clearance zone observed.

Polyethylene Beads:

LDPE granules are the major component of plastic shopping bags which is one of the widely used bags for carrying items; therefore, these fungal strains were experimented with LDPE beads to check their degradation capability. The LDPE bead gets fragmented and depolymerized leading to the formation of smaller molecular weight components involving fatty acids & organic acids (Das and Kumar, 2015; Karamanlioglu et al., 2017). Although few strains had not able to degrade powder particles supplemented solid media, such strains gave meagre 1-4% weight-loss in LDPE beads after eight weeks of incubation (Table.8).

Among all strains of *Aspergillus* sp. only four strains gave positive results in this screening experiment, *A. flavus* (SA2) and *A. fumigatus* (SA3) failed to degrade polyethylene beads. Verma and Gupta (2019) found 14.3% weight-loss of polyethylene beads by *A. flavus* after 4 months. *A. flavus* (SA2) strain brought about only 2.57% weight loss after two months of incubation and which is almost negligible amount of degradation.

Filamentous fungus *A. oryzae* has been reported to be involved in polyethylene degradation. This fungal species can achieve the maximum degradation in 90 days of incubation (Konduri et al., 2010) and the species has potential to reduce the weight of ultraviolet-exposed LDPE up to 51% (Konduri et al., 2011). Maximum weight-loss (7.70%) was witnessed in LDPE beads experimented with *A. oryzae* (SA5). 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 up to 5.37% in a month.

Table.8. Percentage weight-loss of experimented LDPE Beads

Sr. No.	Fungal strain	4 weeks	6 weeks	8 weeks
1	Control	0±0	0±0	0±0
2	*Aspergillus tubingensis*	0±0	5.68±0.17	6.75±1.99
3	*Aspergillus flavus*	0±0	0±0	2.57±0.28
4	*Aspergillus fumigatus*	0±0	0±0	0±0
5	*Aspergillus niger*	5.57±0.33	2.90±0.14	4.41±0.32
6	*Aspergillus oryzae* SA5	5.37±0.33	5.50±0.7	7.70±0.02
7	*Trichoderma reesei*	0±0	0±0	1.38±0.96
8	*Trichoderma viride*	0±0	1.28±0.81	0±0
9	*Trichoderma* sp.	0±0	1.19±0.68	0±0
10	*Pennicillium oxalicum*	0±0	0±0	1.38±0.96
11	*Pennicillium chrysogenum*	0±0	0±0	1.19±0.68
12	*Pennicillium citrinum*	0±0	1.38±0.9	0±0
13	*Rhizopus* sp.	2.70±0.45	2.90±0.17	3.77±1.40
14	*Pestalotiopsis* sp.	0±0	0±0	0±0
15	*Curvularia* sp.	0±0	0±0	0±0
16	*Aspergillus oryzae* SA15	2.70±0.45	2.90±0.17	5.05 ± 0.15
17	*Pleurotus sajorkaju*	0±0	0±0	0±0
18	*Fusarium solani*	2.67±0.15	5.14±0.28	6.92±2.14
19	*Phanerochaete chrysosporium*	0±0	0±0	0±0
20	*Flavodon* sp.	2.79±0.33	4.06±1.81	0±0

(Data is statistically significant as p value was < 0.05)

LDPE beads screened with strains of *Trichoderma* sp. and *Penncillium* sp. showed degradation upto 1.38%. No weight-loss was determined in LDPE beads experimented with *Pestalotiopsis* sp., *Curvularia* sp. and *P. chrysosporium. Flavodon* sp. degraded 2.79% and 4.06% of polyethylene bead in four and six weeks of incubation.

A. tubingensis, A.oryzae SA5 & SA15 were able to degrade 6.75%, 7.70% & 5.05% of LDPE beads, while SA17 showed 6.92% of degradation in eight weeks at room temperature. This preliminary experiment concludes potentiality of these three strains to degrade polyethylene material. Analysis of data proved that the displayed weight-loss values are statistically significant as found p value was < 0.05. Consequently, these fungal strains could be regarded as proficient for the breakage of this complex polymeric structure. The results of this experiment can provide valuable insights into the strains' ability to degrade LDPE and their potential for addressing plastic waste issues.

To confirm the degradation of LDPE beads, FTIR spectroscopy and E-SEM analysis were conducted. These analytical techniques provide valuable information about the changes in the chemical structure and surface morphology of the LDPE beads. Among the experimented fungal strains, *A. oryzae* SA5 exhibited the highest weight loss, indicating a potential degradation capability. To validate this finding, the LDPE bead with maximum weight-loss was analyzed for further confirmation.

In comparison to control polyethylene bead (without fungal treatment) (Figure.13-A) and *A. oryzae* SA5 treated bead, cracks and scratches had been witnessed on the surface formed due to fungal hyphae penetration. (Figure.13- B, C). Surface fractures, bond scratching and change in color can be considered as polymer degradation; changes in texture on surface explains the spread of microbial growth (Mahalakshmi et al., 2012).

Devi et al. (2015) revealed biofilm formation on polyethylene (HDPE) and surface deformation as a result of fungal degradation. Two unknown species of *Aspergillus* were reported as capable strains for polyethylene degradation via biofilm formation on the surface of film. Figure.13 (arrow) evidently represents changes in topography (cracks & scratches) of the surface and microbial growth of *A. oryzae* SA5 on LDPE bead resulting into the degradation.

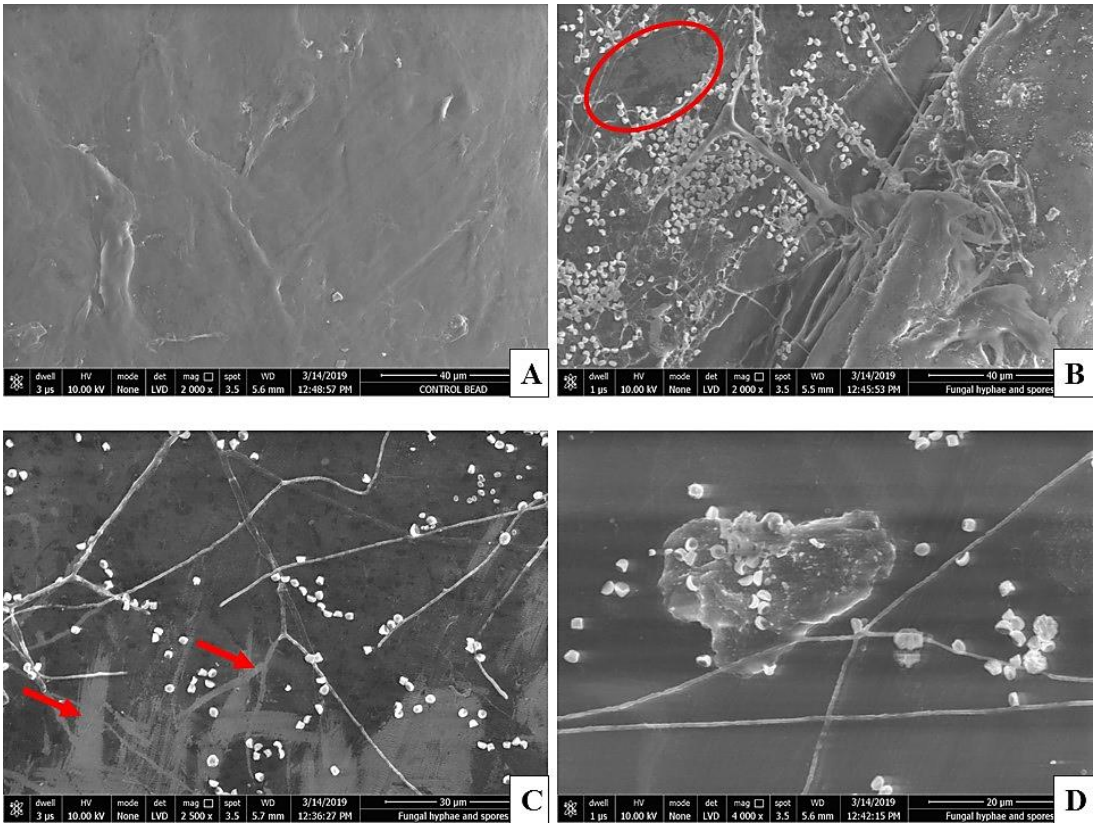

Figure.13. Scanning Electron Microscopic (SEM) images of polyethylene (LDPE) beads: A-Control LDPE Bead (2000x); B, C, D-LDPE bead treated with *A. oryzae* SA5 showing fungal accumulation, cracks & scratches (circle & arrow- B & C) (2000x, 2500x, 4000x)

FTIR analysis was carried out of the LDPE beads which were treated with the fungal strains. FTIR spectra of untreated LDPE bead showed intense peaks at 2915, 2847 and 1462 cm^{-1}, in which 2915 and 2847 cm^{-1} may be representing vibrations of C-H bonds while remaining peak at 1462 cm^{-1} probably depicts C-C bond stretching. Muhonja et al. 2018 reported bands at 720–724 cm^{-1} showing a rocking deformation of experimented LDPE sheets. Similarly, spectra of sample screened with *A. oryzae* SA5 showed an increased peak intensity in the range of 1000-500 cm^{-1} wavenumber

(Figure.14-B). *A. tubingensis* treated LDPE bead spectra showed disappearance of intense peaks at 2915, 2847 & 1462 cm^{-1} which indicates the occurrence of depolymerization.

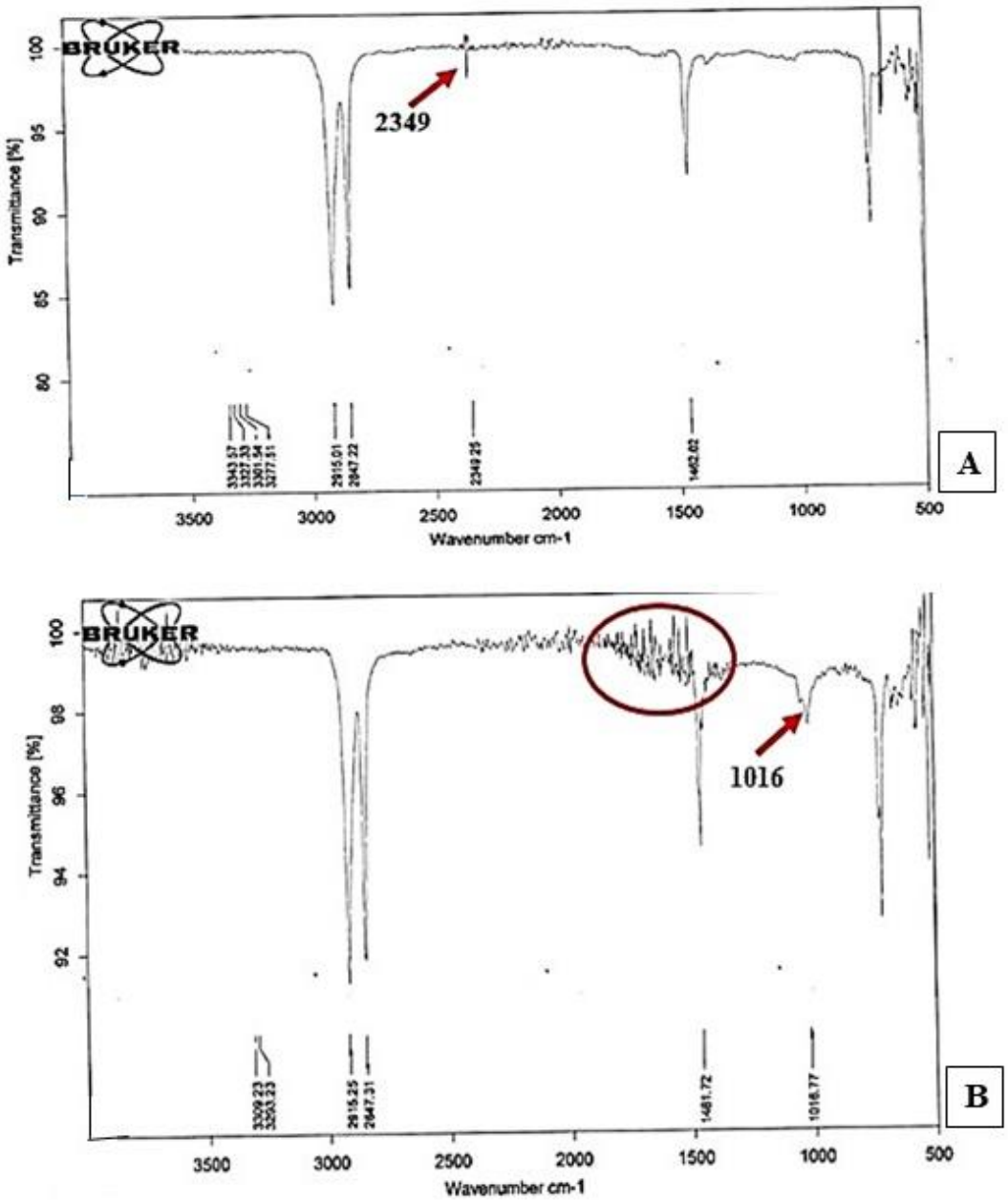

Figure.14. FTIR spectra of polyethylene (LDPE) beads: A-Control LDPE Bead; B- LDPE bead treated with *A. oryzae* SA5

FTIR spectrum of polyethylene treated with *Penicillium simplicissimum* and *Fusarium* sp. showed formation ethers (1018 cm^{-1}) in the study conducted by Sowmya et al. (2015). In the present investigation additional peaks at 1016 $cm^{-1}$and disappearance of band at 2349 cm^{-1} in the spectra obtained of *A. oryzae* SA5 treated LDPE beads indicating breakage of polyethylene structure.

Polymer degradation includes oxidation mechanism which results in the formation of carbonylated compounds which could be identified by band examination in 1900-1500 cm^{-1} region.

The polymer decomposition products promote C=O stretching band near 1725cm^{-1}, consisting of some overlapping bands of component (Barbara, 2000). Polyethylene bead experimented with *A. oryzae* SA5 had shown intense peaks in 1900-1500 cm^{-1} region indicating occurrence of degradation occurrence. 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).

A study by Muhonja et al. 2018 stated that fungi are generally better degraders of polyethylene than bacteria. Present study also proves ability of fungal strains to be able to cleave C-H backbone followed by breakage of polyethylene structure.

Hence out of nineteen fungal strains screened three strains indicated a significant potentiality to degrade polyethylene. Although eight species gave positive results in polyethylene powder screening experiments, *A. oryzae* (SA5 & 15) and *Fusarium solani* (SA17) seemed to be more potential in degrading almost all kinds of polyethylene components. Therefore, ten species showed their potential to degrade polyethylene those are *A. tubingensis* (SA1), *A. oryzae* (SA5 & 15), *A. fumigatus* (SA3), *T. viride* (SA7), *Trichoderma* sp. (SA8), *Rhizopus* sp. (SA12), *Pestalotiopsis* sp. (SA13), *F. solani* (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 films:

Screening of pretreated polyethylene film

Ten fungal strains (SA1, SA3, SA5, SA7, SA8, SA12, SA13, SA15, SA17, & SA19) were subjected to testing on photo-oxidized polyethylene (PE) films. The strains that exhibited the highest capacity for degradation were then chosen for screening on thermo-oxidized films. Subsequently, the selected fungal species were inoculated with thermo-chemically oxidized films to assess their capabilities.

After a two-week incubation period, the experiment demonstrated an increase in the weight of the PE films, indicating the deposition of a fungal biofilm on the film surface. This observation was further corroborated by the analysis of the PE film surface using Environmental Scanning Electron Microscopy (ESEM).

1. Photo-oxidized polyethylene film experiment: A total of ten fungal strains were utilized in the experiment, Photo-oxidized PE films of 2×2 cm pieces were assigned for their ability to break down hydrocarbon bonds. Five species (SA1, SA5, SA13, SA15, and SA17) exhibited the capability to degrade the polyethylene film. These strains demonstrated weight loss percentages ranging from 1% to 3.5% over a four-week period, as indicated in Table.9 and Figure.15. On the

other hand, strains SA3, SA7, SA8, SA12, and SA19 were found to have a lower degradation capacity, with only up to 0.34% of the PE film being degraded. Additionally, it was observed that fungal strains SA1, SA3, and SA5 displayed the highest weight loss in the PE film that was pre-treated under UV rays for 3 hours, compared to the film pre-treated for 9 hours.

Table.9. Percentage weight-loss of Photo-oxidized Polyethylene films

Fungus	Treatments								
	Untreated films			3 hrs UV treated films			9 hrs UV treated films		
	4 weeks	6 weeks	8 weeks	4 weeks	6 weeks	8 weeks	4 weeks	6 weeks	8 weeks
SA 1	0.34 ± 0.02	0 ± 0.01	0 ± 0.002	1.67 ± 0.002	0.67 ± 0.002	0.67 ± 0.001	0.34 ± 0.02	0 ± 0.00	0 ± 0.00
SA 3	0 ± 0.0001	0 ± 0.00	0.34 ± 0.002	0 ± 0.00	0.67 ± 0.004	0.34 ± 0.02	0 ± 0.00	0 ± 0.00	0 ± 0.02
SA 5	0.33 ± 0.02	0.34 ± 0.01	1.33 ± 0.02	2.67 ± 0.04	1.67 ± 0.03	2.67 ± 0.05	1.34 ± 0.02	1 ± 0.001	1 ± 0.005
SA 7	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.01	0 ± 0.00	0 ± 0.00
SA 8	0 ± 0.00	0.34 ± 0.001	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	1 ± 0.02	2.34 ± 0.01
SA 12	0.33 ± 0.01	0.34 ± 0.001	0 ± 0.00	0.34 ± 0.01	0.34 ± 0.04	0.34 ± 0.003	0 ± 0.00	0 ± 0.00	0 ± 0.00
SA 13	0.33 ± 0.01	1.02 ± 0.001	1.33 ± 0.04	1 ± 0.001	2.67 ± 0.0001	2.67 ± 0.001	0.34 ± 0.04	0.34 ± 0.02	0 ± 0.00
SA 15	0.66 ± 0.03	1.02 ± 0.01	1.33 ± 0.003	1.34 ± 0.002	2 ± 0.003	2.34 ± 0.004	**3.67 ± 0.02**	3.34 ± 0.01	2 ± 0.01
SA 17	1 ± 0.01	1.02 ± 0.00	1 ± 0.002	1 ± 0.001	1.67 ± 0.02	2 ± 0.003	**3.19 ± 0.03**	2.34 ± 0.02	0.34 ± 0.002
SA 19	0 ± 0.00	0.34 ± 0.002	0 ± 0.001	0 ± 0.00	0 ± 0.001	0 ± 0.0	0 ± 0.00	0 ± 0.00	0 ± 0.00

(Data is statistically significant as p value was < 0.05)

Khan et al. (2017) reports *A. tubingensis* demonstrates the ability to degrade polyurethane material. Devi et al. (2015) stated a weight loss of 6.02 ± 0.2% in a period of one month inoculation by *A. tubingensis*. In the present experiment, *A. tubingensis* (SA1), a degradation of 1.67 ± 0.002% was observed in 4 weeks incubation period, the polyethylene film treated with UV rays for 3 hours. Within 6th and 8th weeks of incubation period, SA1 exhibited a weight loss of 0.67 ± 0.02%. In comparison, SA1 showed only a 0.34% weight loss in both untreated films and films treated with UV rays for 9 hours after four weeks of incubation. Furthermore, no weight loss was recorded after six and eight weeks of incubation for both types of films in the case of strain SA1.

Many fungal species from the genus *Aspergillus*, namely, *A. niger*, *A. flavus*, and *A. oryzae* are usually employed in LDPE biodegradation, due to its ability to freely and abundantly grow in soil and garbage sites (Bosshard., 2015). A study by Kunlere et al. (2019) reported that *A. flavus*

exhibited a weight loss of 3.33% after six weeks of incubation when inoculated without a carbon source in the culture medium. However, El-Shafei et al. (1998) found no significant weight loss caused by *A. flavus* species. Similarly, in the current experiment, strain *A. flavus* (SA3) demonstrated the ability to degrade 0.67 ± 0.004% of the polyethylene film treated with UV rays for 3 hours in six weeks. After eight weeks of incubation, it exhibited a weight loss of 0.34 ± 0.02% in both untreated films and films treated with UV rays for 3 hours. No weight loss was observed in the PE films pre-treated under UV rays for 9 hours.

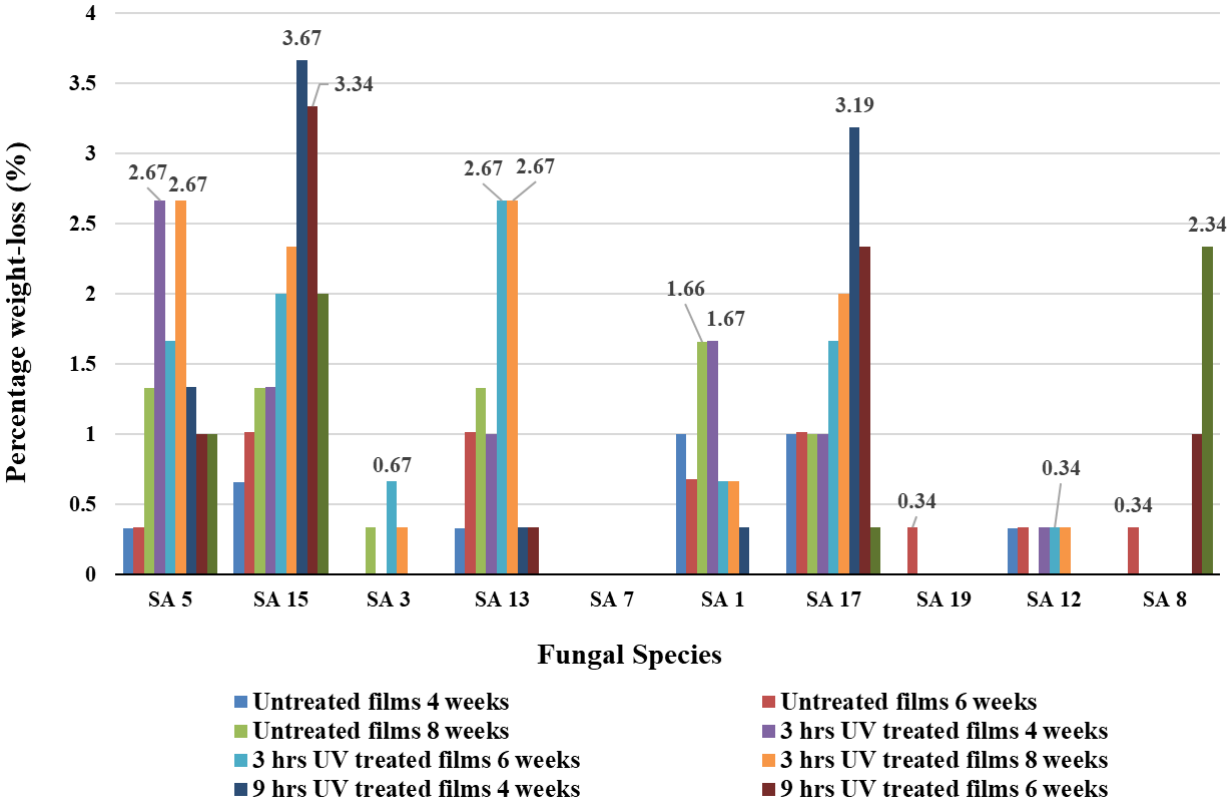

Figure.15. Graph showing percentage weight-loss in Photo-oxidized polyethylene films

In the investigation, two strains of *A. oryzae*, SA5 and SA15, demonstrated variations in their potential for degrading polyethylene films. Konduri et al. (2010) conducted an experiment using UV-irradiated HDPE films for 50 days in soil, which resulted in a recorded weight loss of 72%. This finding supports the idea that pretreatment with UV rays leads to an increased degradation rate. In this investigation, *A. oryzae* (SA5) exhibited the highest weight loss. After one month, it showed a weight loss of 2.67 ± 0.04% in films treated with UV rays for 3 hours. However, after six weeks, it displayed a weight gain of 1.67 ± 0.03%, indicating a change in the degradation trend. On the other hand, films without any pre-treatment and films treated with UV rays for 9 hours exhibited weight losses ranging from 0.33% to 1.34% during the experimental period. This

suggests that the pre-treatment with UV rays for 3 hours led to a higher degradation potential compared to other conditions in the case of strain SA5.

In the study conducted by Konduri et al. (2010), *A. oryzae* was screened using chemically pre-treated followed by UV-irradiated LDPE films for a duration of three months, resulting in a weight loss of 47.2%. Among the fungal isolates, the SA15 strain of *A. oryzae* demonstrated more potential in degrading UV-treated PE film. For films treated with UV rays for 9 hours, SA15 exhibited a weight loss of 3.67 ± 0.02% after four weeks, which remained consistent at 3.34 ± 0.01% after six weeks. After eight weeks, a weight loss of 2 ± 0.01% was recorded. In the case of films treated with UV rays for 3 hours, weight losses of 1.34 ± 0.002%, 2 ± 0.003%, and 2.34 ± 0.004% were observed after 4, 6, and 8 weeks of incubation, respectively. Untreated films displayed a weight loss of 0.66 ± 0.03% after four weeks, and a weight loss of 1.33 ± 0.03% was recorded after eight weeks. Among all the investigated *Aspergillus* strains, *A. tubingensis* and *A. oryzae* strains SA5 and SA15 showed the potential to degrade UV-irradiated polyethylene samples.

Trichoderma viride (SA7) did not exhibit the ability to degrade polyethylene, as it displayed 0% weight loss in all differently treated films. However, it's worth noting that a previous study by Munir et al. (2018) reported a degradation rate of 5.13% in LDPE film by *Trichoderma viride* over a period of 45 days, although this percentage is relatively low. In the present study *Trichoderma* sp. (SA8) also showed no weight-loss, indicating its inability to degrade untreated and 3 hours UV-treated PE films. However, it did demonstrate a weight reduction of 1 ± 0.02% and 2.34 ± 0.01% in films treated with UV rays for 9 hours after six and eight weeks of incubation, respectively. Overall, the *Trichoderma viride* (SA7) strain, *Trichoderma* sp. (SA8) strain, *Rhizopus* sp. (SA12) strain, and *Flavodon* sp. (SA19) strain showed limited or negligible capability to degrade the polyethylene films used in the experiment.

Awasthi et al. (2017) reported a weight loss of 5.92 ± 3% in thermo-oxidized LDPE films by *Rhizopus oryzae*. Here, *Rhizopus* sp. (SA12) and *Flavodon* sp. (SA19) strains displayed negligible weight loss ranging from 0% to 0.34% in films without any treatment and with UV treatment. These findings suggest that the degradation capabilities of different fungal strains can vary significantly, and the outcomes may differ depending on the experimental conditions and specific fungal species involved.

Pestalotiopsis microspora, an endophytic fungus, has been recognized for its potential to degrade polyurethane material, as reported by Russell et al. (2011). In the current study, *Pestalotiopsis* sp. (SA13) fungal isolate demonstrated the highest weight loss in films treated with UV rays for 3 hours. After four weeks of incubation, a weight loss of 2.67% was recorded. Moreover, the strain

was capable of degrading untreated films, resulting in weight losses of 0.33 ± 0.01%, 1.02 ± 0.001%, and 1.33 ± 0.04% after four, six, and eight weeks, respectively. However, it did not exhibit significant weight reduction (0.34%) in films treated with UV rays for 9 hours.

Previous reports have highlighted the capability of the *Fusarium* genus to degrade polyethylene films (Sowmya et al., 2015; Wróbel et al., 2023; Chattopadhyay, 2022). *F. solani*, isolated from a dumpsite, demonstrated significant potential for degrading HDPE films within four weeks, and it was able to reduce 13% of LDPE film within 60 days. In comparison, the *F. solani* (SA17) fungal isolate exhibited significant weight loss in all three treatments. Films without any treatment showed a 1% reduction in weight when subjected to SA17. Films treated with UV rays for 3 hours displayed weight losses of 1 ± 0.001%, 1.67 ± 0.02%, and 2 ± 0.003% after 4, 6, and 8 weeks, respectively. Interestingly, in four weeks, the SA17 strain degraded 3.19 ± 0.03% of UV-treated PE film for 9 hours. However, subsequent biofilm deposition on the film surface resulted in weight gain. Therefore, weight losses of 2.34 ± 0.02% and 0.34 ± 0.002% were recorded after six and eight weeks, respectively.

According to previous reports, UV light treatment has been found to accelerate the degradation of PE degradable plastic films and lead to a loss of their mechanical properties within a relatively short period of two weeks (Johnson et al., 1993; Rana et al., 2022). Taghavi et al. (2021) have highlighted that UV radiation at a shorter distance to the UV source can induce structural changes in plastic, promoting higher microbial colonization and facilitating faster biodegradation processes. This advocates that the proximity of the UV source and the plastic material can impact the degradation rate and microbial activity involved in the biodegradation process. The experiment highlighted the importance of pre-treatments to enhance the breakdown of hydrocarbon bonds, as the fungal strains were only able to degrade up to 1.6% of untreated film within the same week of incubation.

In this research, only five fungal strains demonstrated some degree of degradation, with the SA17 and SA15 strains exhibiting the highest weight losses of 3.19% and 3.67%, respectively. However, it is important to note that after reaching the maximum weight loss, the weight of the film appeared to increase, resulting in a decrease in the observed percentage of weight loss. This phenomenon can be attributed to microbial colonization on the film surface, leading to the formation of biofilms. The presence of biofilms on LDPE samples can contribute to weight loss and is associated with a decrease in the hydrophobicity of the samples, as discussed by Dsouza et al. (2021).

To confirm the potential of the fungal isolates in degrading polyethylene, FTIR analysis was conducted on the films that exhibited the maximum weight loss. The control polyethylene film without any pretreatment of UV rays showed similar intense peaks in the FTIR spectrum.

However, the control film with UV ray pretreatment displayed reduced peak intensities, indicating some changes in the chemical structure of the film, as shown in Figure.16. These changes in peak intensities suggest that the UV pretreatment might have influenced the molecular composition of the polyethylene film, potentially facilitating the degradation process by the fungal isolates.

The spectrum of the untreated film displayed peaks in the region of 2500 cm-1 to 1700 cm-1, indicating the presence of carbonyl groups and alkynes. In the case of pretreated LLDPE film experimented with an isolated bacterium for 40-60 days, a weight reduction of 1.1 ± 0.3 to $3.2 \pm 1.3\%$ was observed, accompanied by a flattening of the carbonyl band in the region of 1300–1100 cm-1, indicating biodeterioration (Ghatge et al., 2020).

Similarly, slight vibrations in the same region were observed in the spectrum of both the untreated and pretreated films (Figure.16-A & B). However, in the film subjected to UV treatment followed by *F. solani* (SA17) fungal biodegradation (Figure.16-C), small peaks were flattened in that region. These observations suggest that the fungal biodegradation process might have influenced the chemical composition of the polyethylene film, leading to changes in the intensity or shape of the peaks related to carbonyl groups and alkynes.

The observed shift of a peak from wavenumber 729 cm^{-1} to 718 cm^{-1} in the fungal-treated film indicates a change in the vibrational behavior of the C-H bonds in the material (Figure.16-C). This shift suggests that the interaction between the fungal activity and the polyethylene film has caused alterations in the chemical environment and molecular structure of the material. Furthermore, the shift of a peak from a higher wavenumber (1471 cm^{-1}) to a lower wavenumber (1461 cm^{-1}) indicates a decrease in bond strength or an increase in bond length of the C-C bonds in the polyethylene sample. This change suggests that the fungal treatment might have induced modifications in the polyethylene's molecular structure, potentially leading to the breakdown of C-C bonds and contributing to the degradation of the material.

The change in Carbonyl Index (CI) is indeed an important factor that indicates the degradation of polyethylene. In the fungal-treated samples, an increase in CI was observed compared to the control (untreated) samples. The polyethylene films used in the experiment also showed changes in CI compared to the control film. While UV treatment did not significantly affect the CI in the control films, the fungal-treated films displayed changes in CI when they were pretreated with UV light for 9 hours.

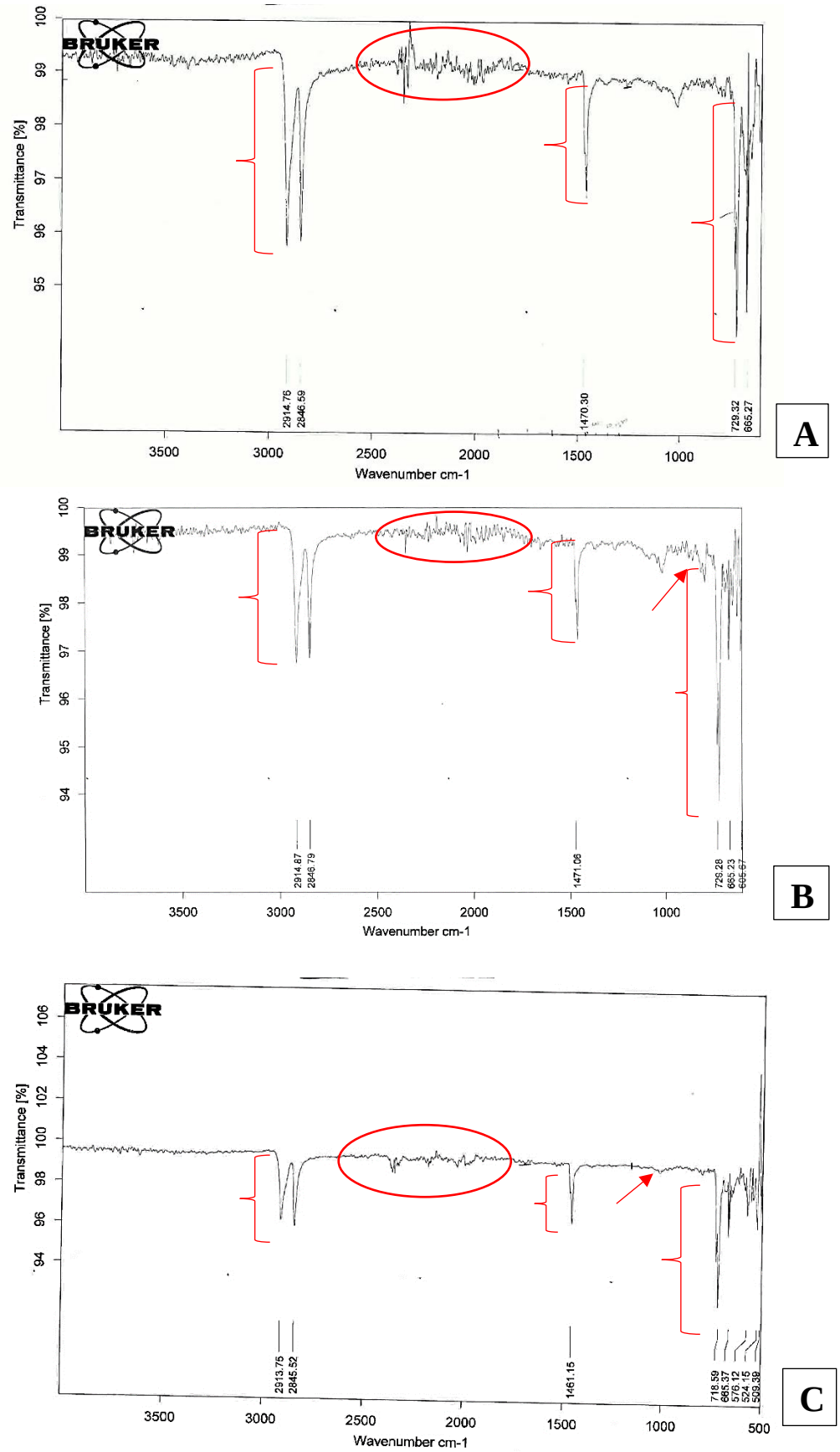

Figure.16. FTIR spectra of photo-oxidised PE films: A-Control (9 hrs UV treated film); B- 9 hrs UV treated film; C- 9 hrs UV treated film experimented with *F. solani* (SA17)

The CI of the control film without any UV treatment was recorded as 0.714. The CI of the control film with 3 hours UV treatment was slightly lower at 0.666, while the CI of the control film with 9 hours UV treatment remained the same at 0.714 (Figure.17). On the other hand, the CI values in the films experimented with SA15 and SA17 strains appeared to be influenced by the pretreatment of UV light for 9 hours. This suggests that the combination of UV pretreatment and fungal degradation might have led to changes in the Carbonyl Index, indicating alterations in the chemical structure and degradation of the polyethylene material.

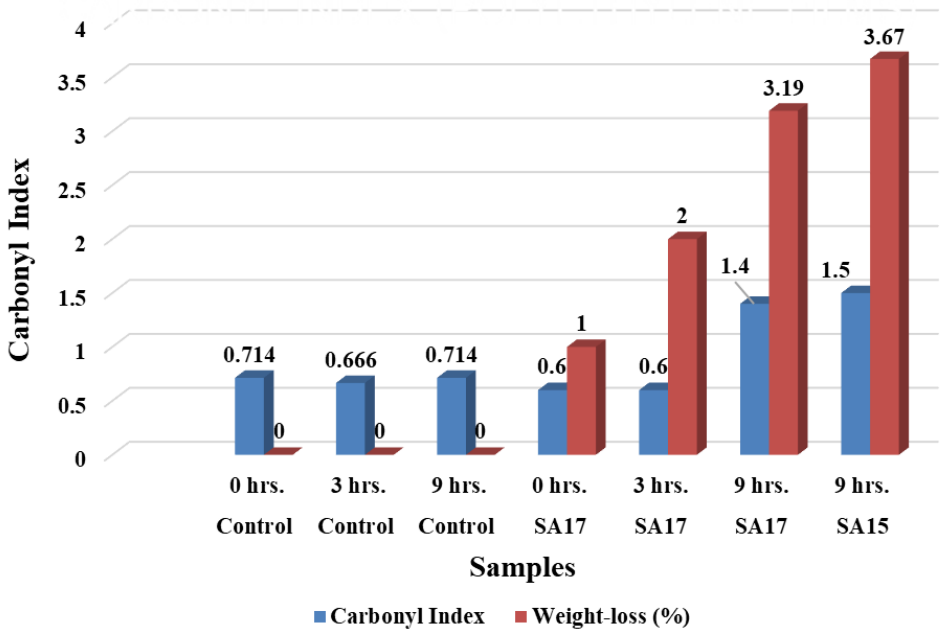

Figure.17. Carbonyl Index of experimented UV treated PE films (SA15- *A. oryzae*; SA17- *F. solani*)

In the experiments conducted with SA17, the untreated PE film showed a CI of 0.6, which corresponded to a weight-loss of 1%. The PE film treated with UV for 3 hours and experimented with SA17 showed a slightly higher CI of 0.6, resulting in a weight-loss of 2%. However, when the PE film pretreated with UV for 9 hours was inoculated with SA17, the CI significantly increased to 1.4, and a higher weight-loss of 3.19% was observed. Similarly, the 9 hours UV pretreated PE film inoculated with SA15 exhibited a CI of 1.5, which correlated with a weight-loss of 3.67%. The higher CI values observed in the UV pretreated films inoculated with fungal strains indicate a greater extent of oxidation and degradation of the polyethylene material.

SEM analysis was conducted to examine the surface morphology of the control film (without any treatment) and the film treated with SA17 fungal strain, which exhibited the highest weight-loss. The SEM images clearly revealed noticeable differences in the topography between the two samples (Figure.18). The control film (Figure.18-A), displayed a smooth, even and regular surface when observed at 1000x magnification. In contrast, the film treated with the SA17 fungus

(Figure.18-B-arrow), exhibited the presence of cracks, cavities, and pits on its surface. These surface irregularities indicate the occurrence of structural damage and degradation caused by the fungal activity, further confirming the impact of fungal degradation on the polyethylene film.

The findings from Spina et al. (2021) and Khruengsai et al. (2021) are consistent with the observations made in this study. *Purpureocillum lilacinum* and *Fusarium falciforme* were reported to induce significant destructions on the surface of polyethylene, such as swellings, pits, and furrows (Spina et al., 2021). Similarly, incubation of LDPE film with *A. niger* resulted in various structural changes, including grooves, cracks, damaged layers, pits, and roughening of the film surface after 30, 60, and 90 days (Khruengsai et al., 2021).

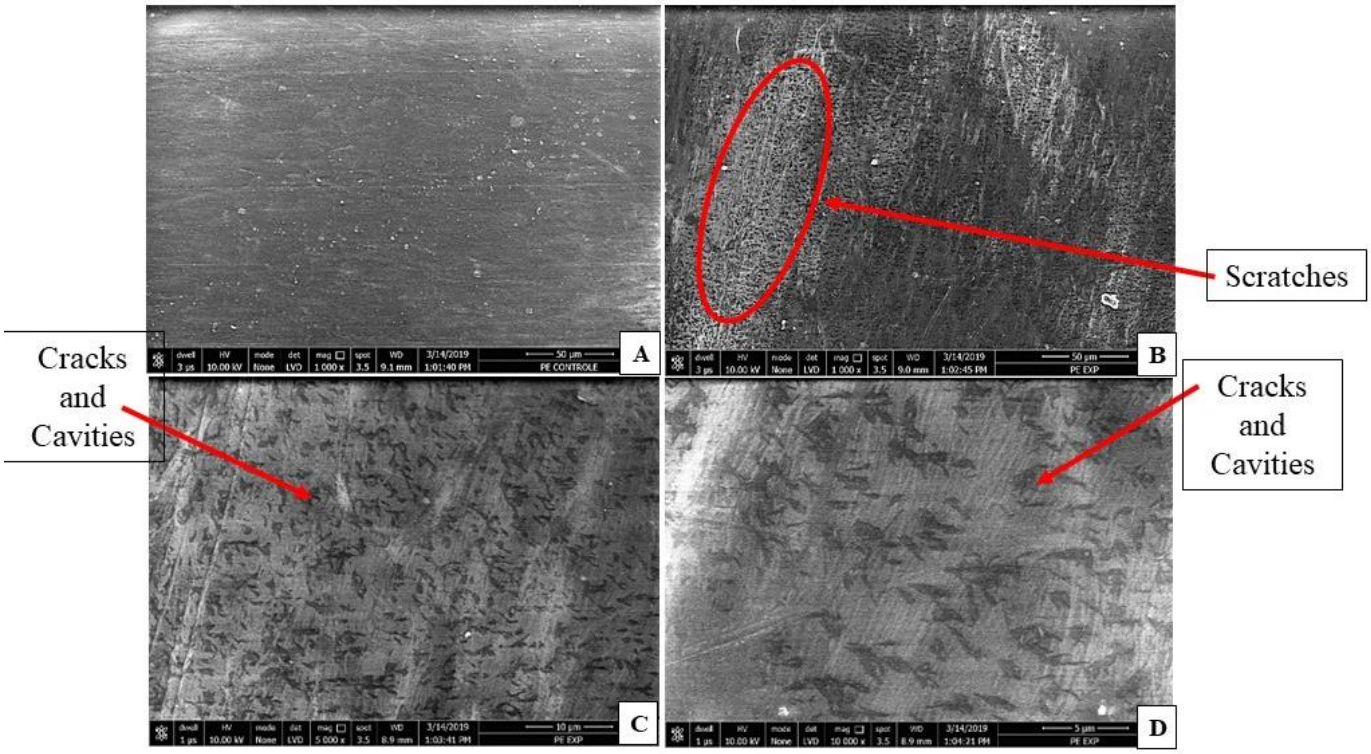

Figure.18. Scanning Electron Microscopic (SEM) images of polyethylene films: A-Control Polyethylene film (1000x); B, C, D- 9 hrs UV treated PE film experimented with *F. solani* (SA17) (1000x, 5000x, 10000x)

F. solani (SA17) also caused notable changes in the surface of the experimented film after 30 days of incubation. Cracks and scratches were observed (Figure.18-C&D). These surface irregularities further indicate the destructive impact of fungal degradation on the polyethylene film, consistent with previous findings in the literature.

The fungal isolates *A. oryzae* (SA5 & SA15), *A. tubingensis* (SA1), *Pestalotiopsis* sp. (SA13), and *F. solani* (SA17) demonstrated significant weight-loss percentages in all three treatments. Among these strains, *A. oryzae* (SA15) exhibited the highest weight-loss in polyethylene films that had been treated with UV for 9 hours and incubated for four weeks. Based on their promising

performance, these five strains were selected for further fungal screening experiments with polyethylene films. It is worth noting that the untreated polyethylene films showed negligible weight loss in all the fungal treatments.

2. Thermo-oxidized polyethylene film experiment: The heat treatment of polyethylene films at different temperatures (45℃ and 70°C) followed by incubation with the five fungal isolates in malt extract culture media for 4, 6, and 8 weeks resulted in significant weight-loss, indicating enhanced degradation compared to untreated films. Figure.19 and Table.10 depicts the weight-loss results obtained from the experiments. The films subjected to heat treatment at 70°C exhibited higher weight-loss percentages compared to those treated at 45℃. This suggests that higher temperatures might accelerate the degradation process of polyethylene.

   Thermal treatment alters the crystallinity level and morphology of the polymer, facilitating biodegradation (Lee et al., 1991). It also leads to a reduction in the size of the polymeric chain, resulting in the formation of new oxidized groups, including carboxyl, carbonyl, and hydroxyl (Sen & Raut, 2015). The findings of this study confirm that thermal treatment resulted in more pronounced degradation of PE films compared to photo-oxidation treatment. Furthermore, it was observed that the degradation rate increased with higher temperatures during the treatment process. In this experiment, untreated films did not exhibit significant weight loss. Weight losses ranging from 5% to 47% were observed in the films treated at 70°C (Figure.19). *F. solani* (SA17) and both strains SA5 and SA15 of *A. oryzae* demonstrated active degradation capabilities. Here also an increase in weight was observed after reaching the maximum weight loss, which is likely attributed to the deposition of fungal and other substances on the film's surface.

Table.10. Percentage weight-loss of Thermo-oxidized Polyethylene films

Fungus	Untreated films			45° Heat pre-treatment			70° Heat pre-treatment		
	2 weeks	**4 weeks**	**6 weeks**	**2 weeks**	**4 weeks**	**6 weeks**	**2 weeks**	**4 weeks**	**6 weeks**
SA 1	0.79 ± 0.001	1.23 ± 0.02	0 ± 0	3.2 ± 0.02	3.7 ± 0.04	1.9 ± 0.01	1.31 ± 0.004	2.63 ± 0.02	0.77 ± 0.01
SA 5	0 ± 0	1.7 ± 0.01	0 ± 0	0.3 ± 0.01	1.28 ± 0.03	1.99 ± 0.02	0 ± 0	6.76 ± 0.02	1.99 ± 0.01
SA 13	0.45 ± 0.001	0 ± 0	0 ± 0	0.39 ± 0.003	1.82 ± 0.02	0.19 ± 0.02	0.28 ± 0.001	3.65 ± 0.01	0 ± 0
SA 15	2.78 ± 0.002	1.49 ±0.03	0 ± 0	3.41 ± 0.02	1.36 ± 0.04	0.18 ±0.003	**9.95 ± 0.002**	2.57 ± 0.03	1.24 ± 0.02
SA 17	4.22 ± 0.01	1.55 ± 0.03	0 ± 0	1.89 ± 0.02	0 ± 0	0 ± 0	**15.12 ± 0.01**	**17.93 ± 0.05**	**17.17 ± 0.02**

(Data is statistically significant as p value was < 0.05)

A. flavus and *A. nidulans*, isolated from a waste recycling site, subjected to preheated PE films at 70°C for a duration of six weeks, resulted in a weight reduction of 1% and 2%, respectively (Ayeni et al., 2022). Balasubramanian et al. (2014) reported a weight loss of 16.7% in four weeks supplemented with *A. terreus*. In the present study, all three strains of *Aspergillus* exhibited significant results, with *A. oryzae* (SA15) showing the highest weight loss among the three strains. 45°C preheated polyethylene films With *A. tubingensis* (SA1) exhibited a maximum weight loss of 3.2 ± 0.02% and 3.7 ± 0.04% after 2 and 4 weeks of incubation, respectively, and after eight weeks of incubation, the weight loss was 1.9 ± 0.01%. Films subjected to 70°C heat treatment showed a weight loss of 2.63 ± 0.02% in four weeks, but interestingly, followed by a weight gain of 0.77 ± 0.01% after six weeks of incubation. In comparison, films without any thermal treatment before fungal experimentation exhibited a weight loss of 0.79 ± 0.001% and 1.23 ± 0.02% in 4 and 6 weeks, respectively.

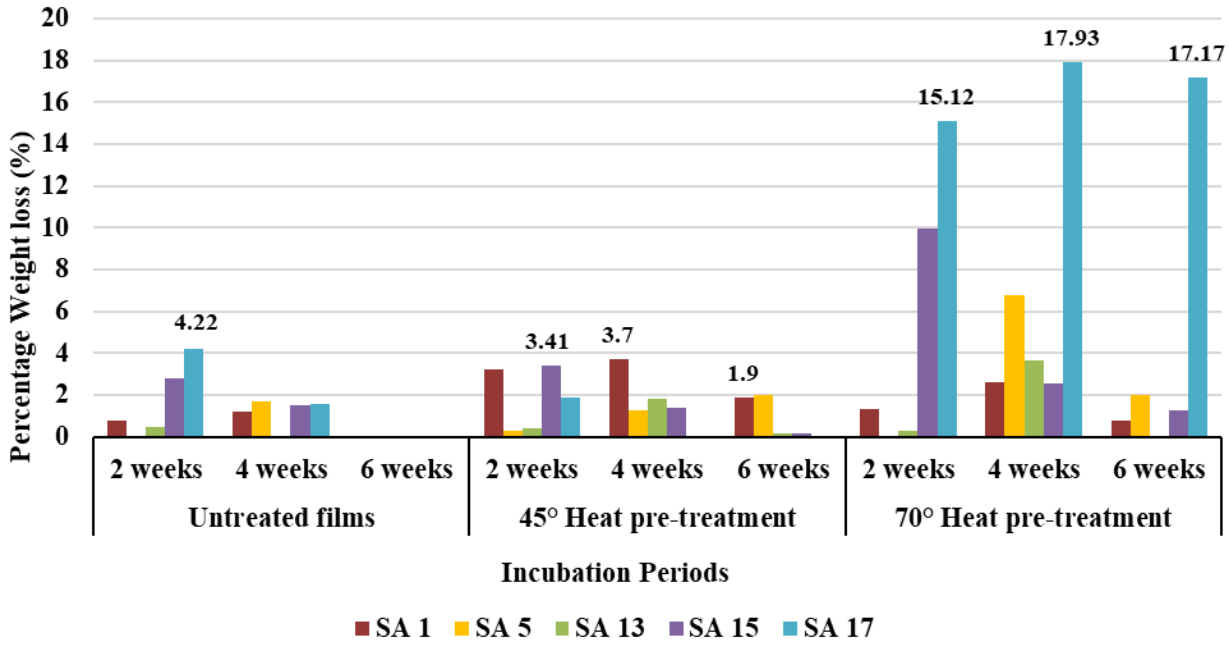

Figure.19. Graph showing percentage weight-loss in pre-heated polyethylene films (SA5- *A. oryzae* (1), SA15- *A. oryzae* (2)., SA13- *Pestalotiopsis* sp., SA1- *A. tubingensis*, SA17- *F. solani*)

Similarly, to the UV pretreated films experiment, both strains of *A. oryzae* showed varying abilities in degrading PE films, with strain SA15 showing greater potential. *A. oryzae* (SA5), significant weight loss of 6.76 ± 0.02% in films pretreated with 70°C heat after four weeks of incubation. After eight weeks, these films showed a weight loss of 1.99 ± 0.01%. The films heated at 45°C showed the maximum weight loss was recorded as 1.99 ± 0.02% after six weeks of incubation. In comparison, untreated films exhibited a weight loss of 1.7 ± 0.01% after four weeks, while no weight reduction was observed in other incubation periods.

A. oryzae (SA15) could reduce 9.95 ± 0.002% of 70°C preheated films within two weeks. However, gradual deposition on the films was observed as the weight loss percentage decreased to 2.57 ± 0.03% and 1.24 ± 0.02% after 4 and 6 weeks, respectively. On the other hand, the 45°C preheated PE films showed less weight loss, with a reduction of 3.41 ± 0.02% within two weeks. Subsequently, a weight gain was observed, with 1.36 ± 0.04% and 0.18 ± 0.003% weight loss recorded after 4 and 6 weeks, respectively.

Pestalotiopsis sp. (SA13) demonstrated significant weight loss in 70°C preheated films, with a maximum reduction of 3.65 ± 0.01% after four weeks of incubation. A weight loss of 0.28 ± 0.001% was recorded within two weeks. In the case of 45°C preheated films, weight losses of 0.39 ± 0.003%, 1.82 ± 0.02%, and 0.19 ± 0.02% were observed after 4, 6, and 8 weeks, respectively. It is worth noting that no previous studies have reported experiments with *Pestalotiopsis* species screened with thermally pretreated PE films.

Sowmya et al. (2015) reported a weight loss of 0.7% in untreated films inoculated with *Fusarium* sp. Pre-treated HDPE film subjected to *F. solani* showed weight losses of 2.65% in 60 days and 2.58% in 90 days. In our experiment (Rani et al., 2020). SA17 strain exhibited the maximum weight loss among all five fungal species. After 14, 30, and 42 days of incubation, weight losses of 15.12 ± 0.01%, 17.93 ± 0.05%, and 17.17 ± 0.02%, respectively, were recorded with 70°C preheated PE films. Surprisingly, in this experiment, SA17 degraded a greater amount of untreated PE films compared to 45°C preheated films. After two weeks, a weight loss of 4.22 ± 0.01% was observed in untreated PE films, while only 1.89 ± 0.02% weight loss was observed in 45°C preheated films.

The FTIR analysis of the treated PE films revealed changes in the peaks and shifts in the wavenumbers corresponding to specific functional groups. The spectrum of the heat-treated PE film subjected to *F. solani* (SA17) exhibited a decrease in peak intensity at 2920 cm^{-1}, indicating reduced stress on the C-H bonds. Additionally, increased peaks were observed at 1630 cm-1 and 1367 cm^{-1} wavenumbers, indicating the formation of carboxylic groups. This is illustrated in Figure.20-B.

The FTIR analysis of the control spectrum revealed two small peaks at 1559 cm^{-1} and 1539 cm^{-1}, which disappeared in the spectrum of the fungal-treated film. These peaks are attributed to the asymmetric stretches of the lead carboxylate species (Stacey et al., 2018). Additionally, the vibrations in the region between 2400-1800 cm^{-1} appeared to be flattened in the fungal-treated spectrum. Furthermore, several peaks at 3445, 2920, 2850, 1019, and 669 cm^{-1} were observed to shift to 3443, 2921, 2851, 1029, and 672 cm^{-1}, respectively, indicating minor changes in the composition.

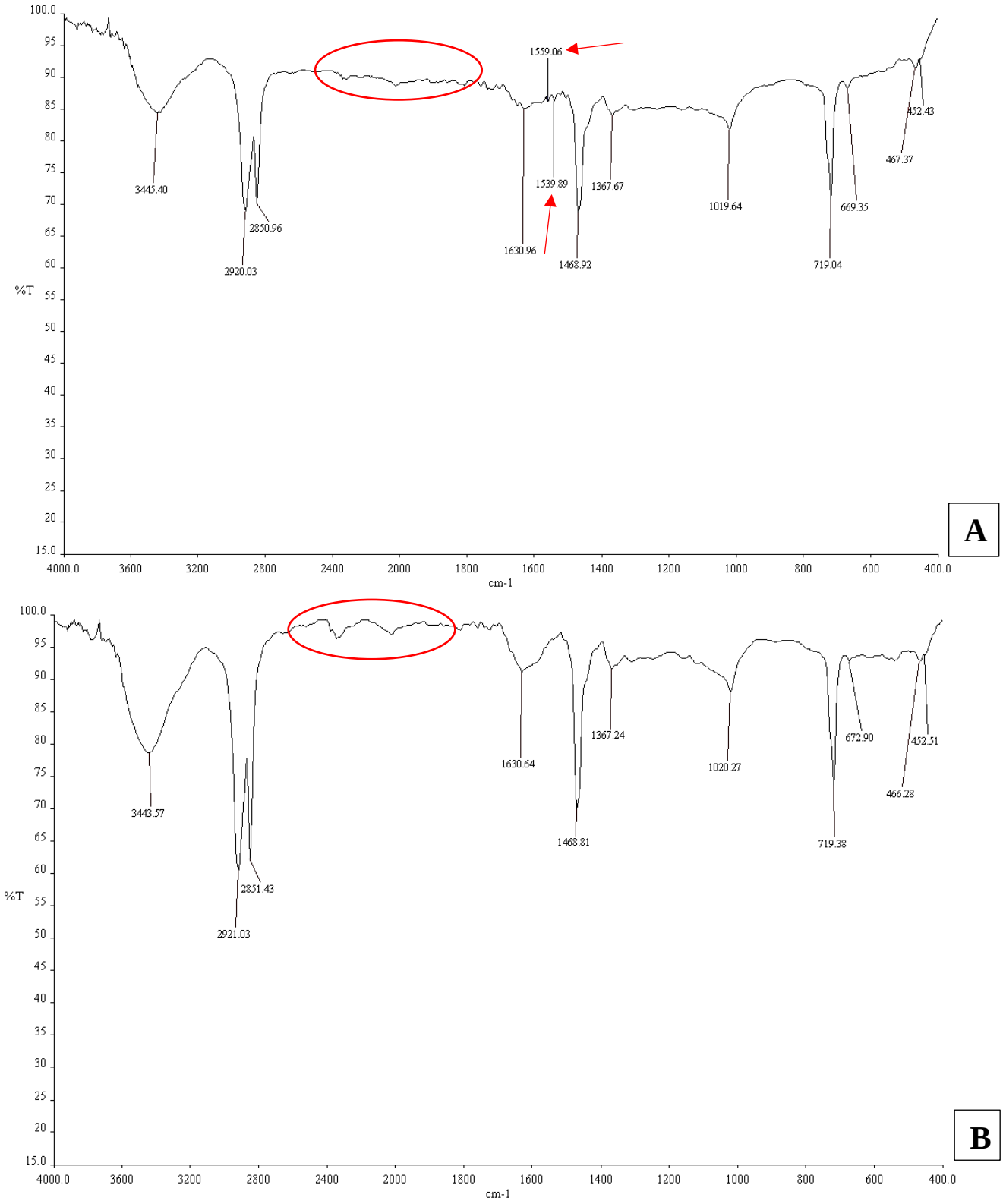

Figure.20. Fourier transform Infrared (FTIR) spectrum of films heat treated PE film experiment (A- Control PE film (arrows-disappearing peaks); B- heat treated PE film experiment with *F. solani*) (circle- peaks with intensity)

SEM analysis was performed on PE film to verify the presence of fungal colonization and degradation on the surface. After four weeks of incubation, the freshly removed film was examined under SEM before undergoing washing with SDS. Figure.21-A, B, and C clearly demonstrate the

colonization of *F. solani* (SA17) on the PE surface. Furthermore, the penetration of fungal hyphae into the surface is prominently visible in Figure.21-B&C at magnifications of 1600x and 3000x. The penetration of fungal hyphae has resulted in distinct patterns on the surface, as depicted in Figure 18-B.

In line with the findings of Mathur et al. (2015), the initial attack on the PE film typically involves surface colonization by the fungus. Furthermore, Costa-Orlandi et al. (2017) reported that *A. terreus*, when colonizing the surface of polypropylene samples, can proceed to degrade the material by forming biofilms. To investigate the surface destruction caused by SA17, the film was washed with SDS and examined under SEM. The analysis revealed the presence of holes, cracks, and scratch-like patterns on the film surface, clearly indicating the degradation that occurred within four weeks (Figure.21-D, arrow).

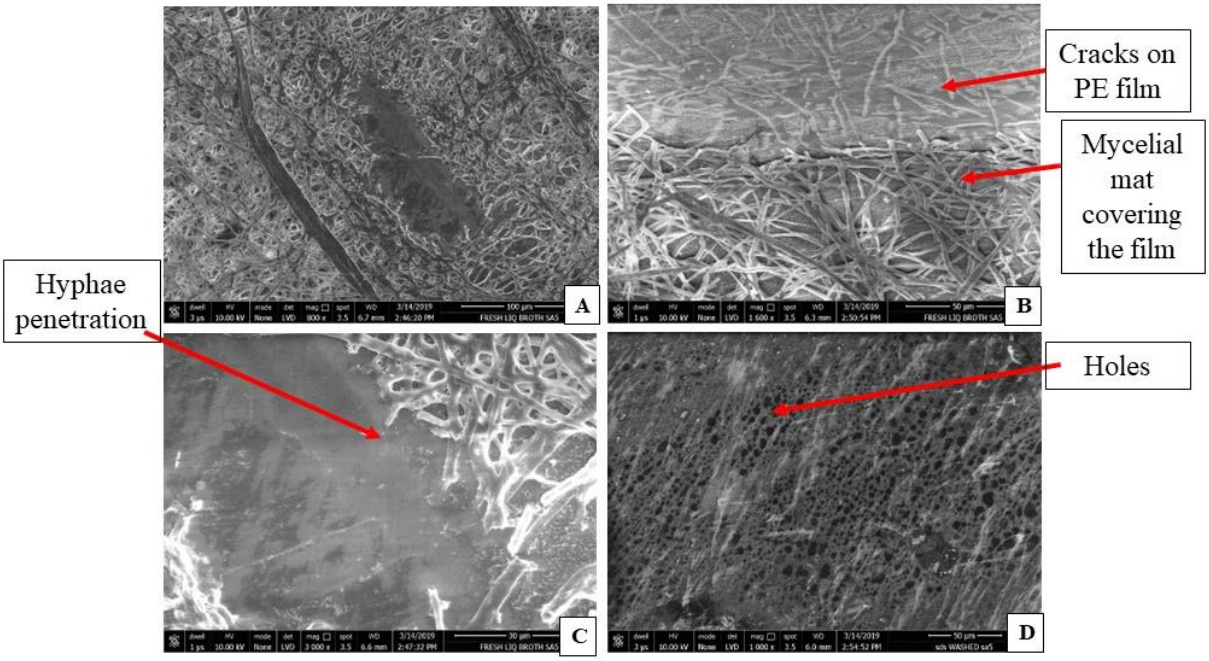

Figure.21. Scanning Electron Microscopic (SEM) images of experimented PE films with *F. solani* (SA17): A- Fungal mycelial mat on PE surface film (800x); B, C- Fungal hyphae network penetrating in PE surface (1600x, 3000x); D- Washed PE films displaying holes and scratches(1000x)

The present experiment demonstrates that a thermal pretreatment at 70°C enhances the microbial degradation of polyethylene. *A. oryzae* (SA15) and *F. solani* (SA17) strains showed remarkable ability to degrade a substantial amount of PE film, evidence of which was supported by FTIR and SEM analysis, which revealed changes in the chemical composition and surface destruction of the films. Based on these promising results, these two strains *A. oryzae* STR2 and *F. solani* (SA15 and SA17) were selected for further screening experiments.

3. Thermo-chemical oxidized polyethylene experiment: In the final screening experiment, a soil+mulch culture medium was selected to test the degradation potential of fungal species in a

natural growth medium, which is important for future practical applications. In this experiment, *F. solani* (SA17) and *A. oryzae* (SA15) were studied using pre-weighed and pretreated polyethylene films in a mixture of soil and mulch under simulated laboratory conditions. The polyethylene films were pretreated with heat followed by nitric acid treatment. After specific incubation periods of 2, 4, and 6 weeks, the films were removed and washed. The results of the weight loss analysis indicated the potential of *F. solani* (SA17) to degrade 21.33% of the nitric acid-treated polyethylene film in just two weeks, even without the presence of a sole carbon source. Pathak and Kumar (2017) reported a significant impact of nitric acid treatment on polyethylene prior to microbial degradation. This suggests that the pretreatment with nitric acid in this experiment may have facilitated the degradation process, enhancing the ability of fungi to break down the polyethylene film.

The literature survey suggests that abiotic degradation of polyethylene can benefit the subsequent biodegradation process by initiating the cleavage of C-H bonds in the polymer. In an investigation conducted by Sangale et al., polyethylene films treated with nitric acid followed by UV treatment exhibited a significant weight loss of 58.51% when experimented in liquid media under constant shaking conditions for eight weeks. In contrast, in the current investigation using a natural growth medium, the highest weight loss was observed after two weeks. However, it is important to note that a phenomenon similar to that observed in the previous study occurred in this experiment as well, where the weight loss percentage decreased after reaching a maximum, indicating a possible weight gain of the film.

Table.11. Percentage weight-loss of Thermo-chemically oxidized Polyethylene films

Incubation Period	Control			SAF17			SAF15		
	Untreated	70° heat	Nitric acid	Untreated	70° heat	Nitric acid	Untreated	70° heat	Nitric acid
2 weeks	0.05 ± 0	0.06 ± 0	0.5 ± 0	5.77 ± 0.001	15.23 ± 0.01	**21.33 ± 0.01**	3.66 ± 0.02	3.84 ± 0.002	16.48 ± 0.01
4 weeks	0.05 ± 0	0.05 ± 0	0.16 ± 0.001	5.15 ± 0.001	9.68 ± 0.004	**17.45 ± 0.02**	2.58 ± 0.01	1.01 ± 0.001	15.38 ± 0.005
6 weeks	0 ± 0	0.06 ± 0	0.17 ± 0	4.98 ± 0.003	9.92 ± 0.02	**18.3 ± 0.01**	0.33 ± 0.001	0.62 ± 0.001	6.25 ± 0.04

(Data is statistically significant as p value was < 0.05)

Figure.19 and Table.11 provide clear evidence of the stimulating impact of abiotic degradation prior to fungal degradation. In an experiment by Chaudhary et al. (2022), LDPE films treated with nitric acid and then experimented with *Cephalosporium* species showed a weight loss of 13% over an eight-week period. In this investigation, *F. solani* (SA17) demonstrated the highest weight loss of 21.33 ± 0.01% in films with nitric acid pretreatment when incubated for two months. The percentage of weight loss observed to be reduced in four and six weeks, indicating possible weight gain in the films. Specifically, the fungus showed a reduction in weight of 17.45 ± 0.02% and 18.3 ± 0.01% in four and six weeks, respectively. *Fusarium* sp. degraded only 0.7% of pretreated

polyethylene in the same time frame (Sowmya et al., 2015), while SA17 had displayed its potentiality to degrade significant amount of polyethylene in two weeks.

Results indicate that heat treatment alone had a lesser impact on the biodegradation of polyethylene compared to the combination of heat and nitric acid treatment. The SA17 showed weight losses of 15.23 ± 0.01%, 9.68 ± 0.004%, and 9.92 ± 0.02% in polyethylene films treated at 70ºC after 2, 4, and 6 weeks of incubation, respectively. Films without any pretreatment displayed lower weight losses of 5.77 ± 0.001%, 5.15 ± 0.001%, and 4.98 ± 0.003% when incubated with SA17 for 2, 4, and 6 weeks, respectively.

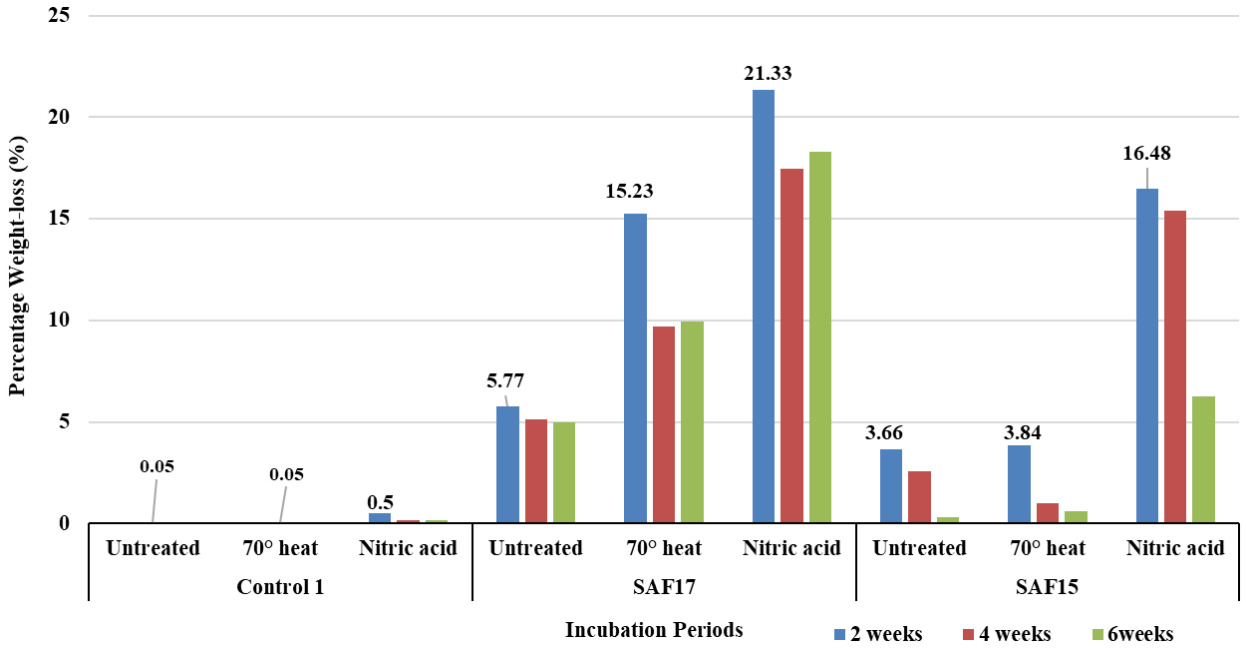

Figure.22`. Percentage weight-loss of Polyethylene films experimented in a mixture of soil and mulch

A. oryzae (SA15) exhibited lower potential for degrading polyethylene films in the soil+mulch growth medium. In a previous investigation with *A. oryzae*, a maximum weight loss of 58.45% was achieved after 3 months when the polyethylene was chemically treated for 2 days followed by UV irradiation for 50 minutes before fungal treatment. In comparison, untreated polyethylene showed only a 6.3% weight reduction during the same test period (Konduri et al., 2010).

The maximum weight reduction observed with SA15 was 16.48 ± 0.01% in films with nitric acid pre-treated when cultured in the soil+mulch growth medium. After four and six weeks of incubation, weight losses of 15.38 ± 0.005% and 6.25 ± 0.04% were recorded, respectively. When the films were subjected to 70ºC heat treatment, weight losses of 3.84 ± 0.002%, 1.01 ± 0.001%, and 0.62 ± 0.001% were observed after two, four, and six weeks, respectively. For untreated films, SA15 could degrade 3.66 ± 0.02%, 2.58 ± 0.01%, and 0.33 ± 0.001% in two, four, and six weeks, respectively.

In the experiment, control replicates of untreated films were included to assess the weight reduction in films without any fungal inoculum, serving as a baseline for comparison. For films without any pretreatment, a negligible weight loss of 0.05 ± 0% was recorded after two weeks, and no weight loss was observed after six weeks. Similarly, films subjected to 70 ºC heat treatment showed a minimal weight loss of 0.06 ± 0% after two weeks. In contrast, nitric acid-treated films displayed slightly higher weight reductions, with 0.5 ± 0%, 0.16 ± 0.001%, and 0.17 ± 0% weight loss after 2, 4, and 6 weeks of incubation, respectively.

The results indicate that SA17 demonstrated higher potential for degrading polyethylene films compared to SA15. SA17 exhibited a weight loss that was 2.11% higher in films without any treatment, 11.39% higher in films subjected to 70 ºC heat treatment, and 4.85% higher in nitric acid-treated films when compared to SA15. To further investigate the degradation process, FTIR and SEM analyses were conducted on the nitric acid-treated films before and after the fungal experiment.

The FTIR spectra analysis of the nitric acid-treated PE film (Figure.23-A) compared to the untreated PE film (Figure.23-A) showed significant changes in peak positions and the appearance of new peaks. Some notable observations include disappearance of peaks at 3448 cm^{-1} (O-H; N-H), 1559 cm^{-1}, 1539 cm^{-1}, 1367 cm^{-1}, and 1019 cm^{-1} in the spectra of nitric acid-treated PE film. This suggests compositional changes in hydroxyl, ether, and carboxylic functional groups present in the polymer structure. The spectra of PE film experimented with SA17 showed the disappearance of peaks at 1260 cm^{-1} and 798 cm^{-1}, indicating the breakdown of C-H bonds. A new peak at 2232 cm^{-1} was observed, which indicates the presence of carbon dioxide (CO_2). This suggests the release of CO_2 gas as a byproduct during the degradation process. The region of 1800-1600 cm^{-1} exhibited significant vibrations, indicating the formation of new aromatic compounds. This suggests

the generation of aromatic compounds as a result of the degradation process.

The observed peak shifts in the FTIR spectra of the nitric acid-treated PE film, such as 2913 to 2914 cm^{-1}, 2845 to 2846 cm^{-1}, 1461 to 1472 cm^{-1}, 718 to 729 cm^{-1}, and 616 to 665 cm^{-1}, suggest subtle changes in the molecular environment. The slight changes in peak positions could be indicative of alterations in the surrounding molecular environment, which can occur due to chemical modifications and interactions during the degradation process. Chatterjee et al. (2010) reported the occurrence of a CH_2 peak at 718 cm^{-1}, while Sangale et al. (2019) observed a reduction in the peak to 720.88 cm^{-1} and 721.05 cm^{-1} with different Aspergillus strains.

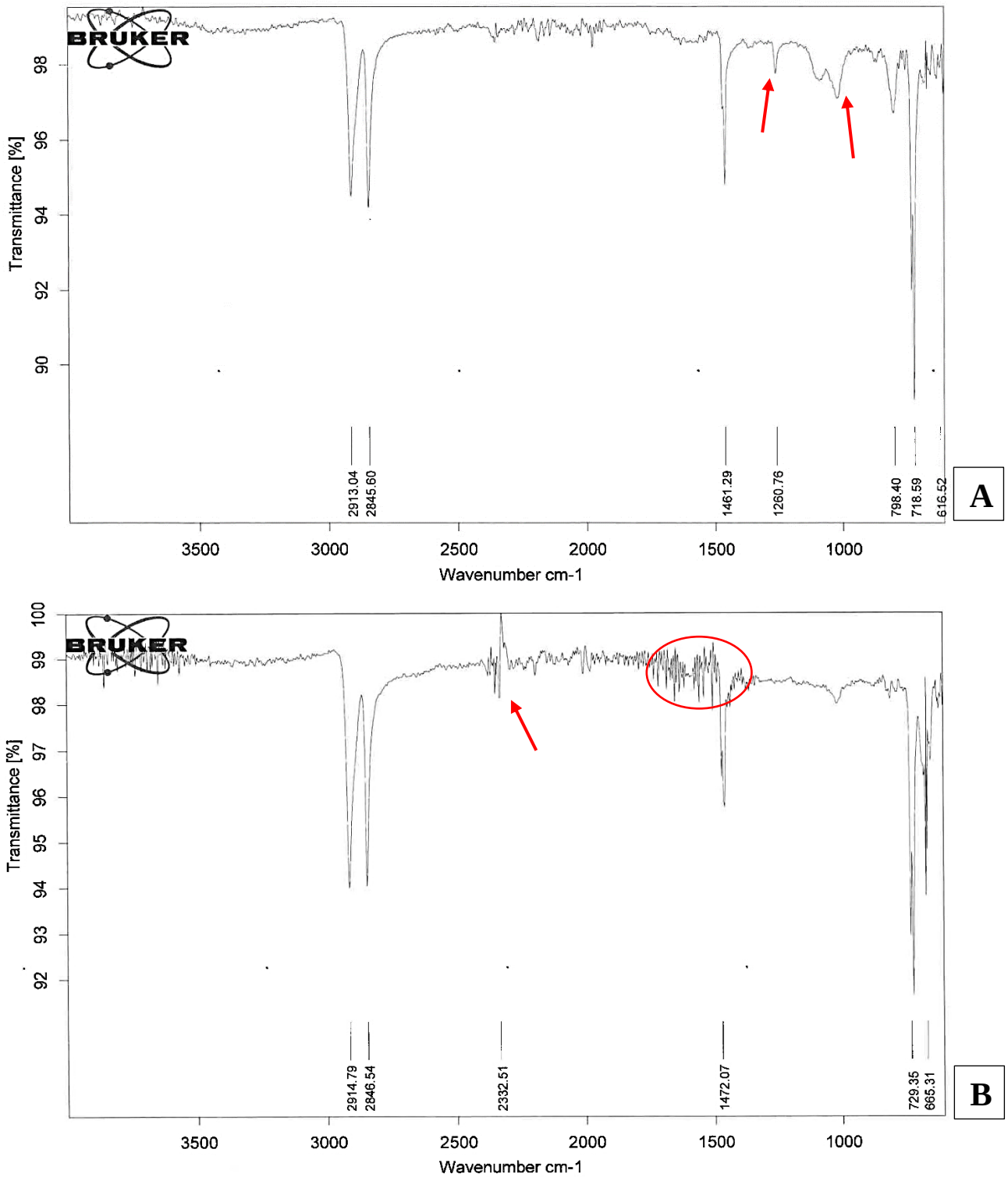

Figure.23. Fourier transform Infrared (FTIR) spectrum of films Nitric Acid treated PE film experiment (A- Control PE film with Nitric Acid treatment (Arrow- disappearing of peaks); B- Nitric Acid treated PE film experiment with *F. solani* (Circle- Vibration in the region, arrow- new peak formation)

The shift from 718 cm^{-1} to 729 cm^{-1} could be indicative of water-related interactions and changes in hydrogen bonding. The presence of water molecules or alterations in hydrogen bonding networks can affect peak positions in the FTIR spectra. Presence of fungal biomass or metabolic byproducts: The presence of fungal biomass or metabolic byproducts on the film's surface can

contribute to peak shifts and changes in the FTIR spectra. The interactions between the film and the fungal components can lead to modifications in peak positions and intensities. Disappearance, formation, and shifting of peaks in samples after microbial treatment indicates the effective usage disintegrate pre-treated HDPE by microorganism (Chaudhary et al., 2023).

The SEM analysis of the PE film experimented with SA17 (Figure.24) revealed various surface modifications and degradation features. These observations are consistent with previous studies that have reported similar degradation effects on the surface of polyethylene films. The SEM images showed the presence of cracks, grooves, and scratches on the surface of the PE film. These surface irregularities are indicative of the physical degradation caused by the action of SA17. The formation of cracks and grooves can be attributed to the breakdown of polymer chains and the removal of material from the film's surface.

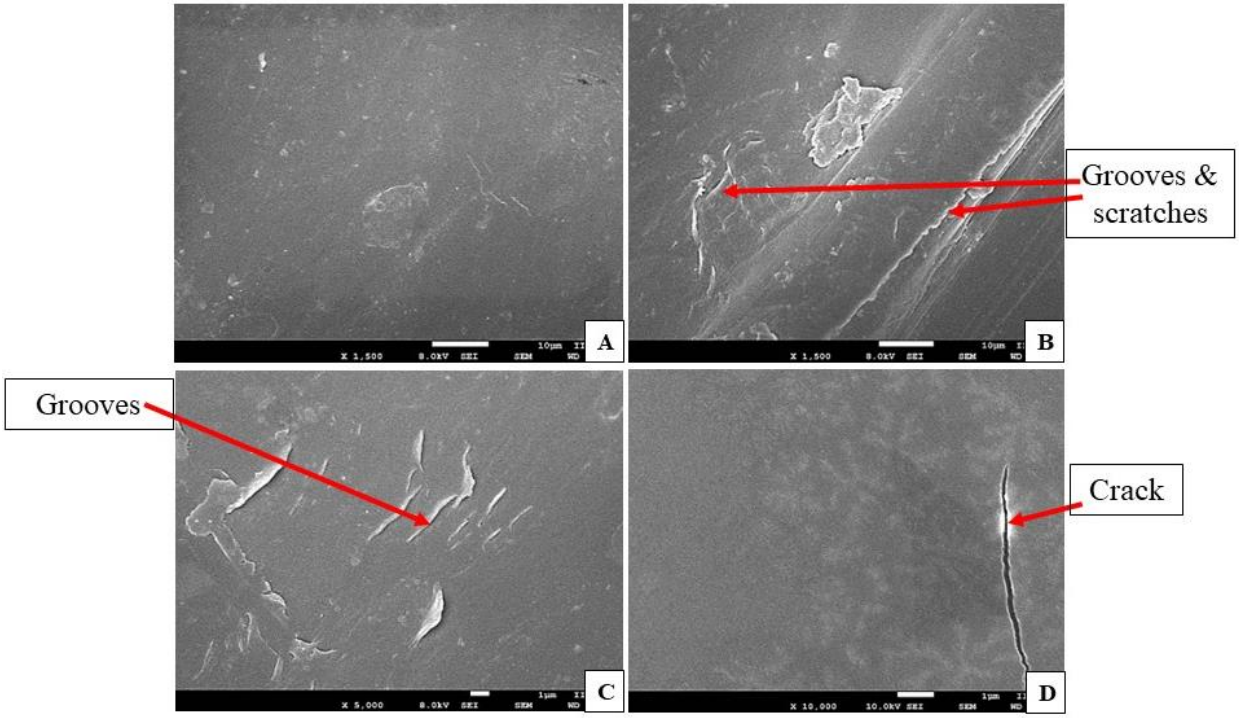

Figure.24. Scanning Electron Microscopic (SEM) images of experimented PE films with *F. solani* (SA17): A- Nitric Acid Treat PE film (1500x); **B, C, D-** Nitric acid treat PE film experimented with *F. solani* displaying morphological alterations scratches (arrow-B), grooves (arrow-C) and crack (arrow-D)on (1500x, 5000x, 30000x) surface

Sangale et al. (2019) mentioned erosion-like features on the degraded films, an observation similar to what was noted in the present study. The SEM analysis conducted after washing the film with SDS confirmed the degradation caused by SA17. Figure.24-A displays minor destruction on the surface of PE due to nitric acid treatment. SA17 apparently degraded the film as grooves and scratches like patterns were observed on the surface when observed at 1500x and 5000x magnifications. Multiple cracks were observed on PE film at 30000x (Figure.24-D). The presence

of grooves and scratches on the film's surface at different magnifications provides visual evidence of the action of the fungus on the polyethylene substrate.

Out to nineteen fungal strains, SA5, SA15 & SA17 showed their ability to degrade polyethylene in the screening experiments. Therefore, SA5 & SA15 strains were sent for confirmation of its identity by DNA sequencing which was performed by first Base DNA sequencing Services, Malaysia and SA17 culture was sent to National Fungal Culture Collection of India (NFCCI), Pune. Molecular identification of SA5 & SA15 strains revealed their identity as *Aspergillus oryzae* (Figure.25-A) and SA17 was identified as *Fusarium solani* MN201580.1 (Figure.25-B) and culture has been deposited at National Fungal Culture Collection of India (NFCCI), Pune.

Figure.25. DNA sequence of fungal strains (A- SA5 & 15; B- SA17)

A-

>Aspergillus oryzae ITS region.

AGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTG
CTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCA
CGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGAT
CTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGA
ATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTG
CTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAG
CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTT
GCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAACATA
TCAATAAGCGGAAGAAAAGAAACCAACCGGGATT

B-

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TCTGGCAAGTCGACCACCGTAAGTSAAACCCTCATCGCGATCTGCTTATCTCGGGTCGTG
|||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
TCTGGCAAGTCGACCACCGTAAGTCAAACCCTCATCGCGATCTGCTTATCTCGGGTCGTG
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Key observations of the study

Screening of nineteen fungal strains was carried out to identify potential PE degraders.

- The PE films were pre-treated with UV rays for 3 and 9 hours, and among the strains tested, in SA1, SA5, SA15, SA13, and SA17, promising results were obtained.
- These five strains were further subjected to experimentation with thermally oxidized films (at 45°C and 70°C). From this experiment, it was observed that SA15 (*A. oryzae*) and SA17 (*F. solani* MN201580.1) fungal isolates exhibited significant weight-loss, surface destruction, and chemical changes, indicating the degradation process.
- Following this, the two strains, SA15 and SA17, were screened with thermo-chemical treated films (60°C + Nitric acid) to identify the potential isolates. Among them, SA17 showed significant weight-loss, and the occurrence of degradation was confirmed by SEM and FTIR analysis.

- A protocol involving heat treatment followed by concentrated nitric acid treatment was selected as the optimal method for further investigation on the degradation of polyethylene films using *F. solani* MN201580.1 (SA17).
- *F. solani* MN201580.1 (SA17) culture was chosen for subsequent enzyme evaluation and optimization experiments due to its higher degradation capability observed in these screenings.