CHAPTER 6

In the previous experiments, enzyme activities were evaluated and optimized conditions were determined. Moreover, fungal degradation of polyethylene was enhanced to 17.47% in the same time frame by supplementing chemical substances like tween 80. Subsequently, in the current investigation, polyethylene films were subjected to experimentation within a natural environment to gauge the effectiveness of the established protocol.

Practical experimentation and assessment of fungal biodegradation of polyethylene under natural conditions

The films were experimented using *F. solani* MN201580.1 (SA17) as the degrading agent within a composite medium of soil and mulch, soil+mulch media supplemented with tween 80 while maintaining optimal conditions. To assess the feasibility of this approach, experiment was conducted in two steps: the experiment was tested in culture bottles under lab-simulated conditions, and subsequently, the entire experimental protocol was replicated within an open-field environment. The degradation was confirmed by weight-loss analysis, evaluation of enzymatic activity, Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) analysis. Additionally, advanced analytical techniques, namely Differential Scanning Calorimetry (DSC) and Gel Permeation Chromatography (GPC), were employed for comprehensive assessment of the degraded polyethylene films.

METHODOLOGY

Preparation of culture replicates in solid state medium

The samples were subjected to a heat treatment at 60°C followed by concentrated nitric acid (HNO_3) treatment. These pre-treated and pre-weighed films were then placed aseptically in a mixture of soil and mulch (dried kitchen waste), maintaining a moisture content of 60% using distilled water. The experimental conditions included a consistent temperature of 30°C and a growth medium pH 8. The 30°C temperature was maintained in BOD (Biochemical Oxygen Demand) incubator for the lab-simulated experimentation. While for the open-field experimentation, the environmental temperature was recorded to be 30±2 °C during the time period of spring season (February-March). Similar to previous experiments, the polyethylene strips were layered in the soil-mulch medium, with two 9mm fungal discs on each layer (Figure.43).

Figure.43. Arrangement of the fungal inoculum & film in soil and mulch medium (Experimental medium)

The initial phase of the experiment was conducted in culture bottles with 100 gm of growth medium under controlled lab conditions for 10, 15, 20, and 25 days. The subsequent field experiment was conducted in earthen pots with 2 kg of soil+mulch medium, and the pots were kept for 10- and 20-day intervals. At the end of each incubation period, the samples were removed, and the films were washed with SDS and then dried. The experiment comprised four different setups as outlined below:

	Control 1	Control 2	Experimental set up 1	Experimental set up 2	Experimental set up 3
Pre-treatment	×	✓	×	✓	✓
Fungal Inoculum	×	×	✓	✓	✓
Tween 80	×	✓	✓	×	✓

Determination of the degradation

The responsible enzymes Laccase & MnP, Protease, Lipase & Esterase were quantified under solid state medium to determine the correlation between degradation and microbial enzyme activities. All five enzymes were extracted along with PE films on desired incubation period as mentioned in the Chapter 4 (methodology). Respective enzyme assays were performed as described in Chapter 2 (methodology). The harvested films were further analysed to confirm the occurrence of fungal degradation (Ghatge et al., 2020) as mentioned below:

-The weight-loss percentage was calculated as described in Chapter 2; and films with significant weight-loss records were further analysed.

-Topographic destructions on films were observed by Environmental- Scanning electron microscopy (E-SEM) under FEI Quanta 200 instrument at Centre for Research in Nanotechnology & Science (CRNTS), Indian Institute of Technology – Bombay, India.

-Chemical changes in experimented films were determined by Fourier transform infrared spectroscopy (FTIR) under 3000 Hyperion Microscope with Vertex 80 FTIR System (Bruker, Germany) at Centre for Research in Nanotechnology & Science (CRNTS), Indian Institute of Technology – Bombay, India.

-The change in molecular weight of the films were analysed by Gel permeation chromatography (GPC) under Turbo matrix–40 (Perkin Elmer) at Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Anand, Gujarat, India.

-The change in melting point of polyethylene films was determined by Differential Scanning calorimetry (DSC) under DSC-8000 (Perkin Elmer) at Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Anand, Gujarat, India. degree of crystallisation was calculated using the formula mentioned below:

$$\% \ Crystallinity = \Delta H_m / \Delta H_m^{ref} \times 100$$

(Where ΔHm is the melting enthalpy of the sample and ΔH_m^{ref} is the melting enthalpy of 100% crystalline polymer)

-The reduction in tensile strength of experimented films was tested by Universal testing machine Mechatronic Engineers ME- UTE 20 at Sabar Insulating Kit Centre, Vadodara, Gujarat, India. The tensile strength of the samples was calculated using formula:

Tensile strength= Break load/Area

RESULT AND DISCUSSION

The final polyethylene degradation experiment using the selected fungus *F. solani* MN201580.1 (SA17) was conducted under optimized conditions. The protocol was initially tested in a lab-simulated environment and then in a field area. The experiment demonstrated the potential of fungus to efficiently degrade a significant amount of polyethylene under these optimal conditions. The confirmation of degradation was achieved through various analyses, including SEM, FTIR, GPC, DSC, and tensile strength testing.

Figure.44: A-C- *F. solani* growth in culture bottles containing soil+mulch at optimum conditions in lab-simulated area**; D-** Experiment set-up in earthen pots in the field area

❖ DEGRADATION EXPERIMENT UNDER LAB CONDITIONS

The genus *Fusarium* is known for living in the soil on organic matter for a long time period. The soil-burial method can enhance the practicability of the degradation process. The degradation experiment was meticulously established with parameters maintained at their optimal levels. A supplementation of 0.05% tween 80 was introduced, moreover, 8 pH & 60% moisture level was maintained in soil+mulch media along with 30°C temperature. *F. solani* MN201580.1 displayed significant and rapid growth within this medium, particularly when supplemented with tween 80. As depicted in Figure.44-A&B, visible fungal growth on the PE film became evident after five days, with Figure.44-C showing vigorous *F. solani* MN201580.1 growth on the surface within 10 days of incubation period. The harvested films were at designated incubation intervals removed for the analysis. To determine the significance of enhancer and pretreatment, control replicates were kept without adding enhancer, fungal inoculum and as well as without providing pre-treatments to the film.

Enzyme activities: Enzymes are key factor in the degradation activity, and they were evaluated and optimized in previous experiments. After the stipulated incubation period, PE films were harvested and all the five specific enzyme activities were determined simultaneously. All five enzyme activities were found to be significant during this experiment. Among the four distinct experimental configurations, the experiment set-up with pre-treated films and tween 80 presented noteworthy amount of enzyme activities (Table.26).

In this study, *F. solani* MN201580.1 (SA17) exhibited significant enzymatic activity in experimental set up 3 (Pretreatment + SA17 + tween 80) culture replicates, while control replicates showed negligible activity. Laccase activity was highest on 20^{th} day with a peak at 15.75 ± 0.05 U/ml activity and control replicates showed slightly lower (Figure.45-A and Table.26). Whereas MnP, protease, esterase and lipase activities were reported to be maximum on 15^{th} day in experimental set up 3 (Pretreatment + SA17 + tween 80) with 1.46 ± 0.02 U/ml, 509.5 ± 5.507 U/ml, 599.78 ± 0.56 U/ml, and 218.11 ± 0.4 U/ml, respectively. Comparatively, control 1 and 2 (without fungal inoculum) did not show any enzyme activity, while other experimental set up also showed lower enzyme activities. These findings indicate positive influence of pre-treatment and tween 80 supplementation on the enzyme activity.

F. solani MN201580.1 (SA17) demonstrated the potential for plastic degradation through the production of these enzymes, highlighting its significance in environmental applications. Control setups exhibited some enzyme activity, though lower than the experimental replicates, emphasizing the role of fungal inoculum and inducers in enhancing enzymatic plastic degradation.

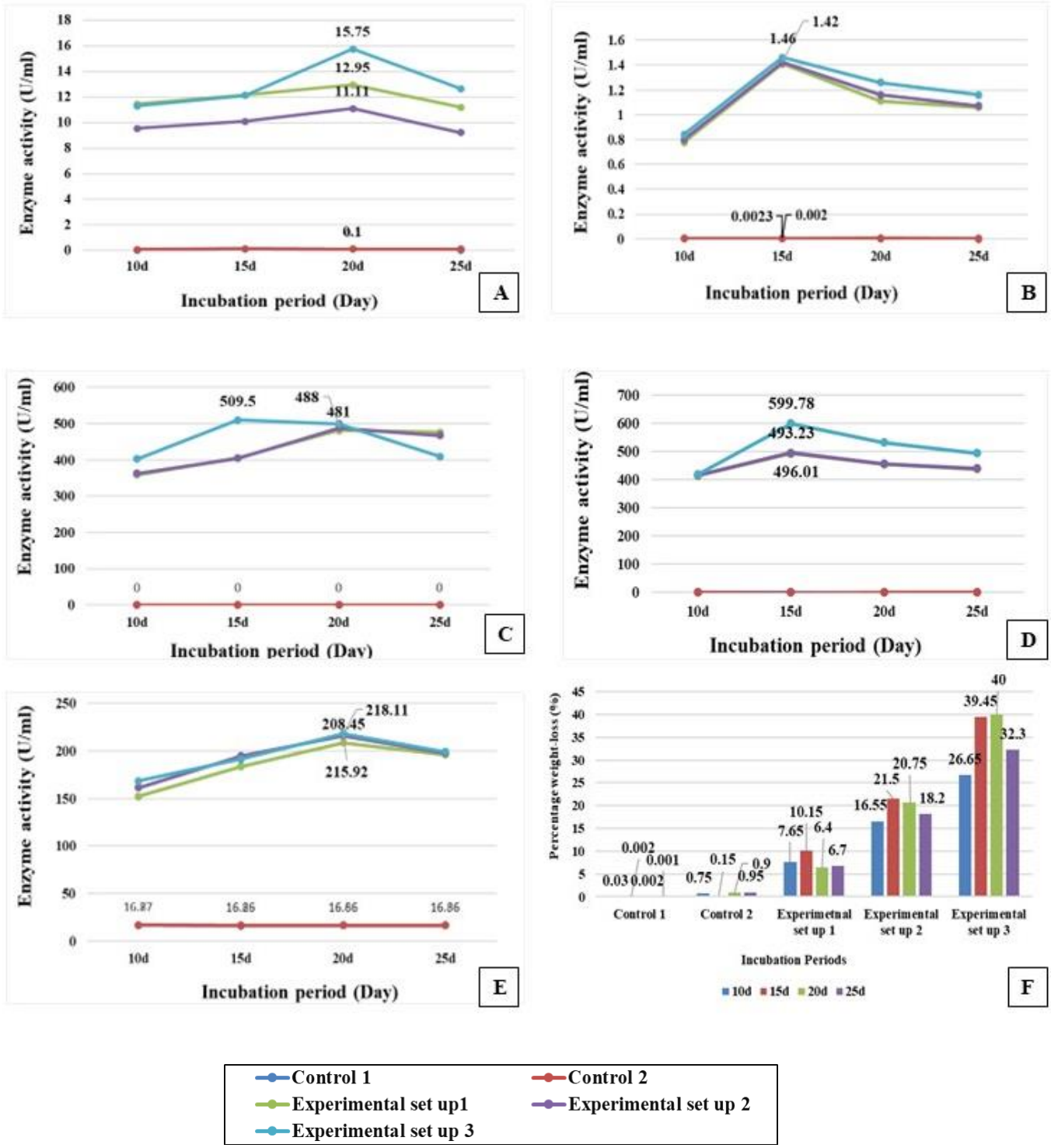


Figure.45. Enzyme activities and weight-loss of PE films in soil+mulch medium in lab-simulated area: A- Laccase, **B-** MnP, **C-** Protease, **D-** Esterase, **E-** Lipase; **F:** Percentage weight-loss of polyethylene films experimented in lab-simulated area at optimum conditions

Table.26. Enzyme production by *F. solani* MN201580.1 in lab simulated condition (Soil+mulch medium)

Enzyme Activity (U/ml)	Incubation Period	Treatments				
		Control 1	Control 2	Experimental set up 1	Experimental set up 2	Experimental set up 3
		(No treatment)	(Pretreatment + tween80)	(SA17 + tween 80)	(Pretreatment + SA17)	(Pretreatment + SA17 + tween 80)
Laccase	10d	0.02 ± 0	0.09 ± 0.02	11.46 ± 0.04	9.56 ± 0.04	11.31 ± 0.22
	15d	0.14 ± 0.01	0.12 ± 0.02	12.15 ± 0.3	10.1 ± 0.04	12.13 ± 0.04
	20d	0.10 ± 0.004	0.10 ± 0.03	12.95 ± 0.07	11.11 ± 0.02	**15.75 ± 0.05**
	25d	0.01 ± 0.04	0.10 ± 0.03	11.2 ± 1.14	9.21 ± 0.03	12.66 ± 0.5
MnP	10d	0.002 ± 0.0004	0.004 ± 0.005	0.78 ± 0.006	0.80 ± 0.01	0.84 ± 0.01
	15d	0.002 ± 0.0001	0.0023 ± 0.005	1.41 ± 0.008	1.42 ± 0.01	**1.46 ± 0.02**
	20d	0.003 ± 0.0001	0.005 ± 0.004	1.11 ± 0.006	1.16 ± 0.01	1.26 ± 0.006
	25d	0.002 ± 0.0004	0.001 ± 0.002	1.06 ± 0.007	1.07 ± 0.006	1.16 ± 0.01
Protease	10d	0	0	358.5 ± 1.91	363 ± 2.58	402.5 ± 3.41
	15d	0	0	405 ± 2.58	404.5 ± 3	**509.5 ± 5.507**
	20d	0	0	481 ± 1.15	488 ± 2.82	499 ± 2.58
	25d	0	0	476 ± 1.63	467 ± 4.76	409.5 ± 1.91
Esterase	10d	0.04 ± 0.003	0.06 ± 0.01	415.17 ± 0.41	416.99 ± 0.48	419.88 ± 0.55
	15d	0.06 ± 0.001	0.05 ± 0.02	493.23 ± 0.21	496.01 ± 0.33	**599.78 ± 0.56**
	20d	0.002 ± 0.0001	0.07 ± 0.01	455.90 ± 0.277	457.38 ± 0.76	533.23 ± 0.46
	25d	0.002 ± 0	0.07 ± 0.01	438.49 ± 0.5	440.42 ± 0.25	494.99 ± 0.50
Lipase	10d	16.77 ± 0.001	16.87 ± 0.01	152.27 ± 0.58	161.38 ±0.27	168.36 ± 0.34
	15d	16.25 ± 0.003	16.86 ± 0.003	183.59 ± 0.47	194.52 ± 0.35	191.18 ± 0.27
	20d	16.66 ± 0.0012	16.86 ± 0.001	208.45 ± 0.43	215.92 ± 0.53	**218.11 ± 0.4**
	25d	16.36 ± 0.003	16.86 ± 0.16	195.9 ± 3.08	197.39 ± 0.27	199.08 ± 3.20

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

Weight-loss: The percentage weight-loss of harvested films was calculated for each incubation and treatment. The most substantial weight loss was noted in the 20 days incubation period, aligning with the period of maximum enzyme activity. Notably, films subjected to pre-treatment exhibited the highest activity levels when supplemented with both fungal inoculum and tween 80.

The highest weight reduction in experimented films was recorded to be 40 ± 0.14% after 20 days in experimental set up 3 (Pre-treatment+ SA17+ tween 80) as seen in Figure.45-F & Table.27. In these replicates, 26.65 ± 0.21% weight-loss was recorded in 10 days, which increased to 39.45 ± 0.77% after 15 days. The percentage weight-loss slowed down after fifteen days and peaked at 20th day. Similar to previous degradation experiments, the films started to gain after certain incubation days

indicating deposition. As reduced percentage of weight-loss was recorded after 25 days (32.3 ± 0.28%).

Table.27. Percentage weight-loss in experimented PE films under lab-simulated area at optimised conditions

Incubation period	Weight-loss (%)				
	Control 1	**Control 2**	**Experimental set up 1**	**Experimental set up 2**	**Experimental set up 3**
	(No treatment)	(Pretreatment + tween80)	(SA17 + tween 80)	(Pretreatment + SA17)	(Pretreatment + SA17 + tween 80)
10d	0.003 ± 0.00002	0.75 ± 0.07	7.65 ± 0.07	16.55 ± 0.07	26.65 ± 0.21
15d	0.002 ± 0.0002	0.85 ± 0.07	10.15 ± 0.07	21.5 ± 0.14	39.45 ± 0.77
20d	0.002 ± 0.0002	0.9 ± 0	6.4 ± 0.07	20.75 ± 0.07	**40 ± 0.14**
25d	0.001 ± 0	0.95 ± 0.07	6.7 ± 0.14	18.2 ± 0.1	32.3 ± 0.28

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

In comparison to experimental replicates, Experimental set up 1 (SA17+ tween 80) showed 29.5% less weight-loss indicating necessity of pre-treatment to the PE film. The films without any pre-treatment presented maximum weight-loss as 10.15 ± 0.07% after 15 days of incubation. In initial ten days, *F. solani* MN201580.1 could degrade 7.65 ± 0.07% of untreated polyethylene. After 20 and 25 days of inoculation, 6.4 ± 0.07% & 6.7 ± 0.14% weight-loss was recorded, respectively.

F. solani MN201580.1 could degrade 21.5 ± 0.14% of pre-treated films in fifteen days when inoculated without adding tween 80 (Experimental set up 2), which is 18.5% less weight-loss than experimental replicates. These pre-treated films 16.55 ± 0.07% degraded after ten days of inoculation, which is higher than control 1 and lesser than experimental replicates. After 20 and 25 days, respectively, 20.75 ± 0.07% and 18.2 ± 0.1% weight reduction was noted.

As expected, control replicates, did not show significant weight-loss. These films were degraded up to 0.95% when incubated for 25 days, which is not very significant and justifies the potentiality of *F. solani* MN201580.1.

Control 1 (no treatment) and Control 2 (Pretreatment + tween 80) did not exhibit significant in absence of fungal inoculum, this indicates degradation efficiency of *F. solani* MN201580.1 These results highlight the effect of amalgamated treatments on the fungal degradation of polyethylene. The pre-treatment of nitric acid accelerated the percentage weight-loss, which was again enhanced when supplemented with tween 80 supplemented. As the pre-treatment of heat and nitric acid help fungus to degrade polymer faster, and tween 80 enhances the enzyme activity and fungal accumulation.

Therefore, the combination of pre-treatment and inducer benefits the fungus to degrade more amount of polyethylene. For further analysis of degradation by SEM and FTIR was performed on films with highest degradation rate.

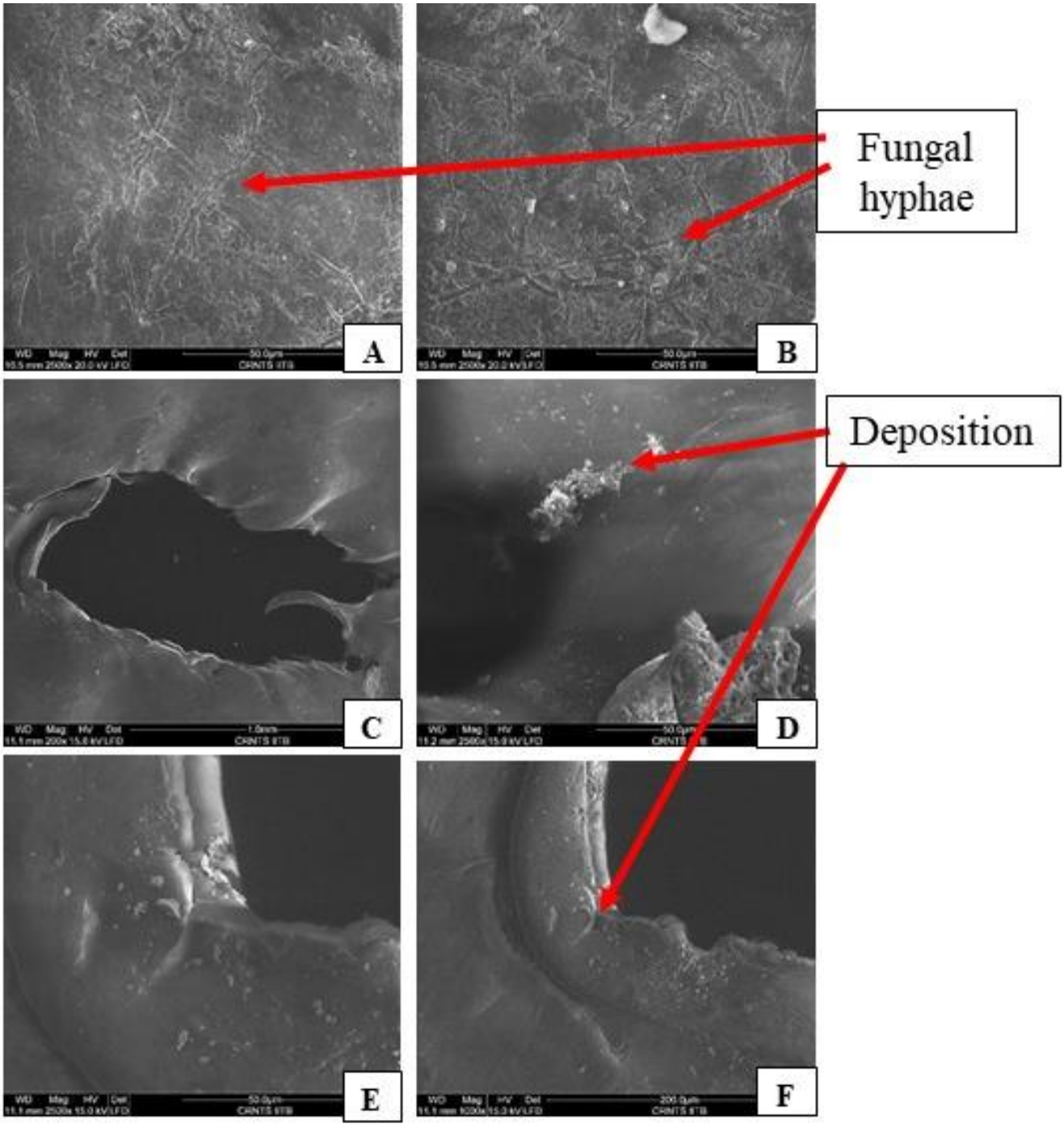


Figure.46. A- Experimented film after 10- & 20-day incubation displaying fungal hyphae penetration, hole and polymer fragments: A & B- Fungal hyphae penetration and cracks on the surface after 10-day incubation(2500x)**; C-** Torn film developing a big hole after 20-day incubation (200x)**; D, E, F-** Magnified images of a hole showing fragmented polymer in the margin (2500x, 2500x, 1000x)

Surface topographical changes (SEM study): The films harvested from experimental set up 3 (Pre-treatment+ SA17+ tween 80) showed more changes on the surface than other replicates. Untreated films (Experimental set up 1) surface was smooth and unhindered but the pre-treated films distinctly showed holes and surface destructions on the films experimented without tween 80. Pre-treatment of nitric acid and supplementation of tween 80 in medium have contributed to notable changes like decolouration, larger holes, crumpled & torn film. Those films were studied under SEM to determine the changes (Figure.46).

Within 10 days of incubation period mycelial mat and hyphal penetration was clearly noticed on the pretreated film experimented with fungus and tween 80 (Figure.46-A & B). The supplementation of tween 80 has resulted in to compact fungal accumulation on the surface. After 10 and 20 days of incubation, visible large holes were observed (Figure.46-C) and the vicinity of the holes at higher magnifications showed deposition of small particles at the margin and gaps (Figure.46-D, E & F). These particles are assumed to be fragmented pieces of polymer.

These observations explain the degradation mechanism by fungus, the fungal accumulation takes place and hyphae penetrates into the film within 10 days. Which creates small holes and film starts degrading, eventually these holes turn into a big hole and the film loses weight. The fragmented particles may get deposited on the film resulting into increased weight-gain in the experimented films.

Changes in chemical properties (FTIR): The degradation changes the chemical composition of the film and may form new components and that was determined by performing FTIR on the films. The film pretreated with fungus along with tween 80 was compared with only pre-treated films (Figure.47-A & B). The spectra of fungal treated showed new peaks at 1630 cm^{-1}, 1575 cm^{-1} and 1538 cm^{-1}. In which 1630 cm-1 represents to the presence of carbonyl group, typically C=C stretch, similar observations were recorded in previous studies (Rajandas et al., 2012; Kim et al., 2023; Da Costa et al., 2018). The peak at 1575 cm-1 indicates stretching vibrations of C=C showing the presence of ketone & aldehyde group (Çepelioğullar & Pütün, 2014). Additionally, two peak shifts to higher wavenumber were observed, 1367 cm-1 to 1368 cm-1 and 1017 cm-1 to 118 cm-1, explains molecule mass reduction.

These peak shifts and new peak formations clearly confirm the occurrence of polymer degradation resulting into new fragmented molecules. This investigation partially proved the applicability of the experiment to degrade polyethylene film at optimised conditions and further experimented in open field area.

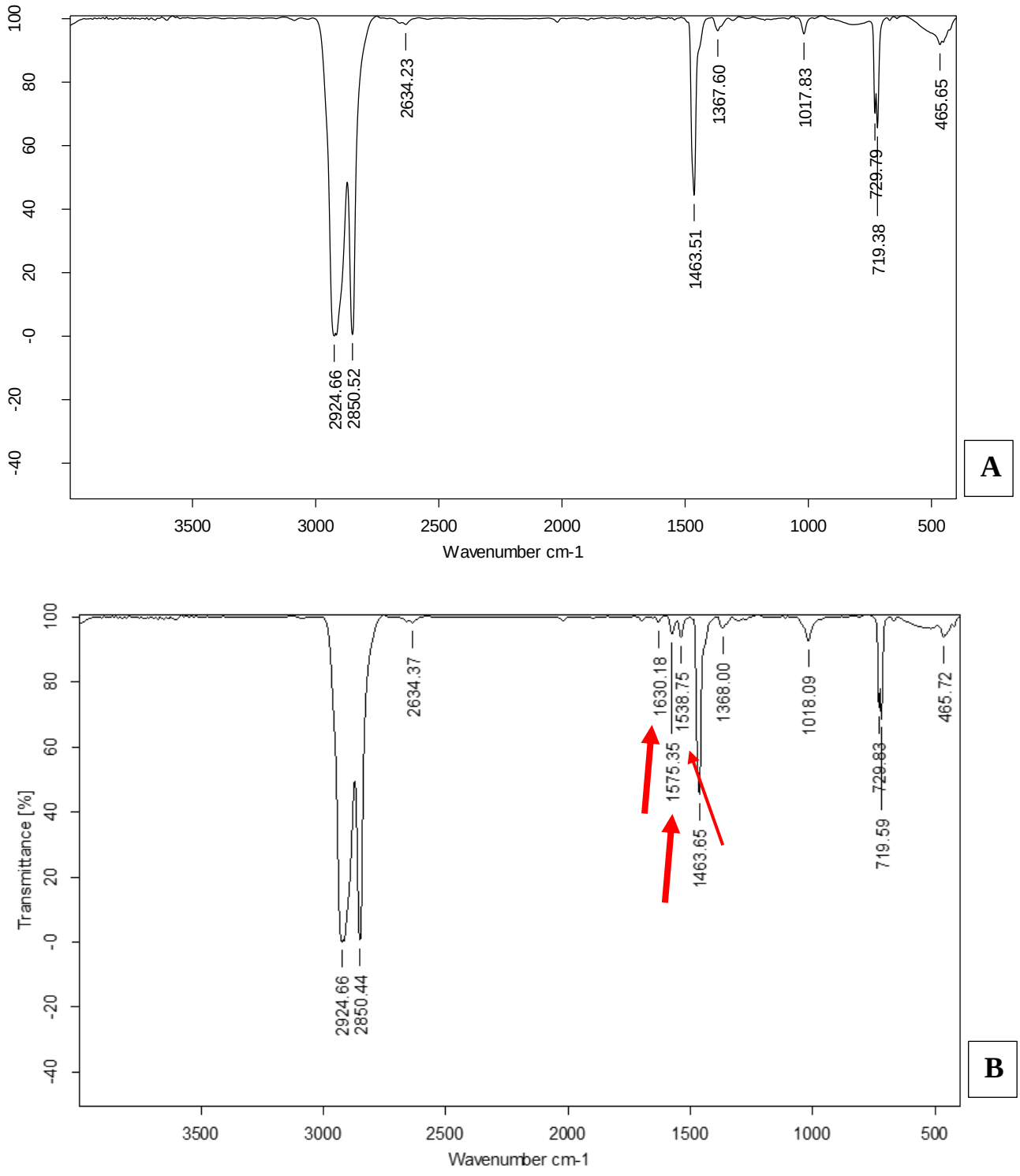


Figure.47. FTIR spectra of experimented PE films in lab-simulated area at optimum conditions: A- Pre-treated film**; B-** Experimented PE film with maximum weight-loss (arrow-formation of new peaks)

❖ DEGRADATION EXPERIMENT AT SIMULATED OPEN FIELD AREA

The experiment was set-up in open field area located in Botanical Garden, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat to test the protocol. Fungal inoculums were added to earthen pots containing soil and mulch along with tween 80, and control replicates were same as the lab simulated set-up. The set-up was executed with experimented monoculture in earthen pots. Earthen pots were selected as simulated open field condition so as to reduce the chances of contamination and to prevent leaching out as well. *F. solani* MN201580.1 (SA17) started growing vigorously within 3 days and by 10th day the growth increased with hyphal growth spread out profusely (Figure.48). The degradation and enzyme activities were recorded to be maximum after 20 days. The films were harvested after incubation periods of 10th and 20th day. The degradation potential was further determined by quantifying enzymes, weight-loss reduction, SEM, FTIR, DSC and GPC.

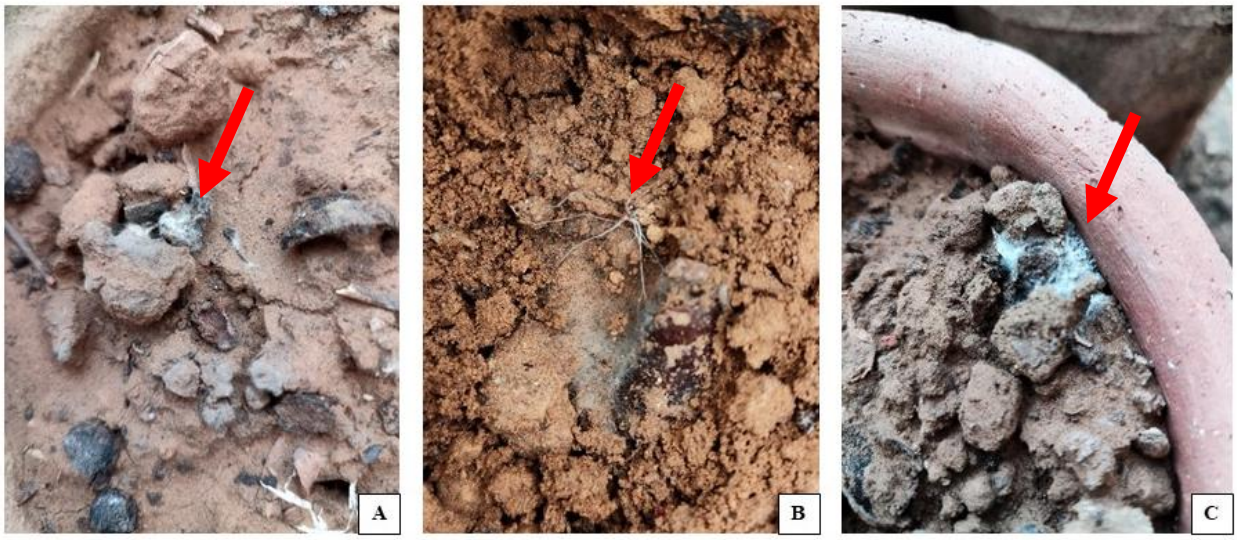


Figure.48. *F. solani* growth in earthen pots containing soil+mulch at optimized conditions in open field area after 10-day incubation

Enzyme activity: All five enzyme activities by *F. solani* MN201580.1 (SA17) were found to be significant and the activity measured appeared to be similar to the lab experiment (Figure.49-A-E and Table.28). The enzyme activities were optimum on the 20th day of the incubation period in experimental set up 3. The maximum activity of ligninolytic enzymes was recorded to be 15.78 ± 0.07 U/ml (laccase) and 1.27 ± 0.008 U/ml (MnP) in experimental setup 3. Experimental setup 3 exhibited significant enzyme activities of protease, esterase, and lipase with values 505 ± 3.41 U/ml, 533.17 ± 0.70 U/ml, and 217.73 ± 0.61 U/ml, respectively. Whereas experimental setups 1 and 2 showed slightly lower enzyme activities. On the 20th day, 12.96 ± 0.09 U/ml (laccase), 1.12 ± 0.008 U/ml (MnP), 482.5 ± 2.51 U/ml (protease), 455.62 ± 0.35 (esterase), and 209.1 ± 0.51 U/ml (lipase) enzyme activities were recorded in experimental set up 1. Experimental set up 2 exhibited 11.10 ± 0.02U/ml (laccase), 1.17 ± 0.01 U/ml (MnP), 487.5 ± 2.51 U/ml (protease), 457.78 ± 0.5 U/ml

(esterase), and 216.27 ± 0.59 U/ml (lipase) activity on 20th day of incubation. In the case of control 1 and 2 replicates, no significant activities were found in the absence of fungal inoculum.

Table.28. Enzyme production by *F. solani* MN201580.1 in soil+mulch medium at open field area

Enzyme Activity (U/ml)	Incubation Period	Treatments				
		Control 1	**Control 2**	**Experimental set up 1**	**Experimental set up 2**	**Experimental set up 3**
		(No treatment)	(Pretreatment + tween80)	(SA17 + tween 80)	(Pretreatment + SA17)	(Pretreatment + SA17 + tween 80)
Laccase	10d	0.02 ± 0	0.09 ± 0.02	11.44 ± 0.03	9.59 ± 0.05	11.41 ± 0.35
	20d	0.04 ± 0.02	0.09 ± 0.02	12.96 ± 0.09	11.10 ± 0.02	**15.78 ± 0.07**
MnP	10d	0.001 ± 0.004	0.004 ± 0.005	0.79 ± 0.01	0.81 ± 0.003	0.85 ± 0.01
	20d	0.001 ± 0	0.004 ± 0.005	1.12 ± 0.008	1.17 ± 0.01	**1.27 ± 0.008**
Protease	10d	0.011 ± 0.01	0.015 ± 0.03	357.5 ± 11	363.5 ± 3	404 ± 1.63
	20d	0.002 ± 0.01	0.005 ± 0.01	482.5 ± 2.51	487.5 ± 2.51	**505 ± 3.41**
Esterase	10d	0.01 ± 0	0.03 ± 0.03	415.89 ± 0.58	416.93 ± 0.45	420.11 ± 0.39
	20d	0.02 ± 0	0.06 ± 0.03	455.62 ± 0.35	457.78 ± 0.5	**533.17 ± 0.70**
Lipase	10d	16.56 ± 0.001	16.86 ± 0.002	152.4 ± 0.54	162.19 ±0.44	168.54 ± 0.41
	20d	16.76 ± 0.002	16.86 ± 0.001	209.1 ± 0.51	216.27 ± 0.59	**217.73 ± 0.61**

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

Similar to the lab-simulated experiment, all five enzyme activities were significant when the experiment was set up at the field area by keeping all parameters at the optimum level. These observations evident the ability of *F. solani* MN201580.1 to produce significant enzyme activities in open field areas.

Weight-loss: The films harvested after ten and twenty days of incubation was thoroughly washed with SDS to remove the debris and other matter present on the film, and further weighed. The highest weight-loss was recorded after 20 days of incubation with 41.5 ± 0.56 percent in experiments set up cultures inoculated with *F. solani* MN201580.1 on pre-treated films supplemented with tween 80 as enhancer (Figure.49-F and Table.29). Within 10 days of incubation 27.05 ± 0.07% weight-loss was obtained.

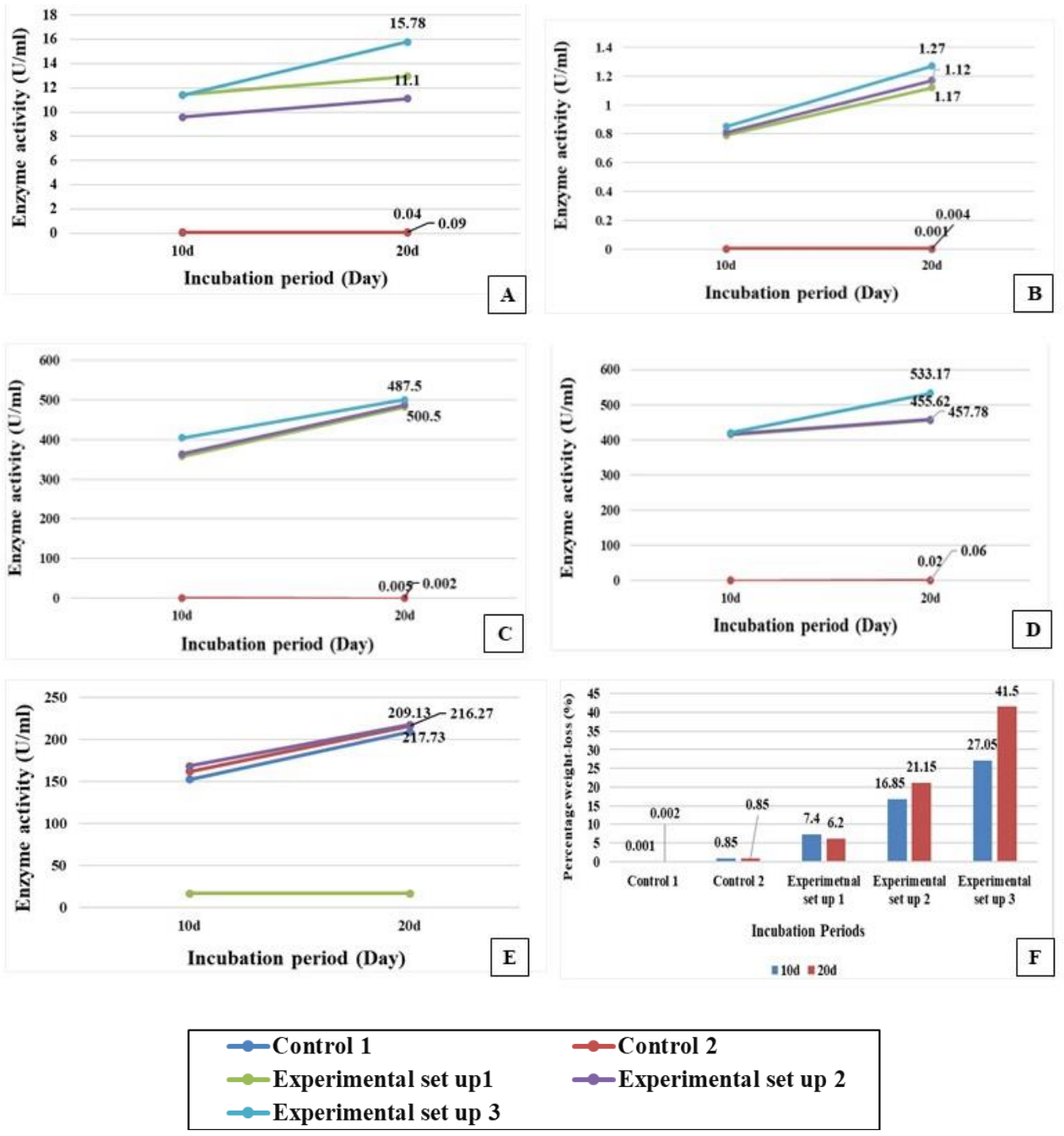


Figure.49. Enzyme activities and weight-loss of PE films in soil+mulch medium in field:A- Laccase, **B-** MnP, **C-** Protease, **D-** Esterase, **E-** Lipase; **F:** Percentage weight-loss of polyethylene films experimented in field at optimum conditions

Experimental set up 1 (SA17+ tween 80) showed 19.65% and 35.3% less weight-loss compared to experimental replicates after 10 and 20 days, respectively. Respectively, on tenth and twentieth day, 7.4 ± 0.14% and 7.4 ± 0.14% weight-loss was recorded in these replicates. While Experimental set up 2 (Pre-treatment+ SA17) showed slightly higher degradation due to nitric acid pre-treatment. These set of treatments exhibited 16.85 ± 0.07% and 21.15 ± 0.07% weight loss in 10 and 20 days of incubation. The replicated did not show any degradation in the absence of *F. solani* MN201580.1.

Similar to previous experiment, control replicates without fungal inoculum did not show significant weight-loss.

Table.29. Percentage weight-loss in experimented PE films in open field area at optimised conditions

Incubation period	Weight-loss (%)				
	Control 1	**Control 2**	**Experimental set up 1**	**Experimental set up 2**	**Experimental set up 3**
	(No treatment)	(Pretreatment + tween80)	(SA17 + tween 80)	(Pretreatment + SA17)	(Pretreatment + SA17 + tween 80)
10d	0.001 ± 0	0.85 ± 0.07	7.4 ± 0.14	16.85 ± 0.07	27.05 ± 0.07
20d	0.002 ± 0	0.85 ± 0.07	6.2 ± 0.14	21.15 ± 0.07	**41.5 ± 0.56**

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

Results of the experiments performed under simulated open area appeared to be same as that conducted under lab conditions. It showed the significance of nitric acid pre-treatment and supplementation of tween 80 in the soil & mulch medium. The occurrence of degradation by *F. solani* MN201580.1 was further tested by studying surface topography, reduction in tensile strength and changes in chemical structure, and additionally physical properties molecular weight & melting point of film.

Surface topographical changes (SEM): The samples with highest percentage weight-loss were observed under SEM to determine the topographic changes after fungal degradation. The films harvested from the experimental set-up 3 (Pre-treatment+ SA17+ tween 80) showed much visible changes than other films. The colour of the films turned slightly pale and surface of the film appeared to be rough. The experimental set up 2 (Pre-treatment+ SA17+ tween 80) replicates also showed rough surface. The similar morphological changes were observed in polyethylene and polypropylene when experimented with *Lysinibacillus* sp. JJY0216 (Jeon et al., 2021). creasing surface roughness. They reported surface roughness on LDPE due to biodegradation and the roughness increased with the incubation period.

Akhigbe et al. (2023) reported the appearance of pinholes, cracks and particles on the surfaces of LDPE films after incubation with bacterial strain *Proteus mirabilis* for 78 days. In the present experiment, tiny holes and scratches begin to develop within ten days of incubation period, (Figure.50-A & B). These minor destructions on the film turned into larger hole in the film as seen in the Figure.50-C. The fungal hyphae of *F. solani* MN201580.1 penetration into the film surface leading to cracks in the film, this phenomenon was evidently observed under SEM (Figure.50-D). Previous reports of polyethylene degradation by *Asperigllus* species have reported surface erosion in the form of cracks and pits (Dsouza et al., 2021; Pramila and Rakesh, 2011; Mahalakshmi et al.,

2012). The other region of the film showed cracks and deposition on the surface (Figure.50- E & F). The SEM observations revealed that topographic changes occurred within 10 days of incubation increasing by the 20th day of incubation confirming the potential degradation of polyethylene by *F. solani* MN201580.1.

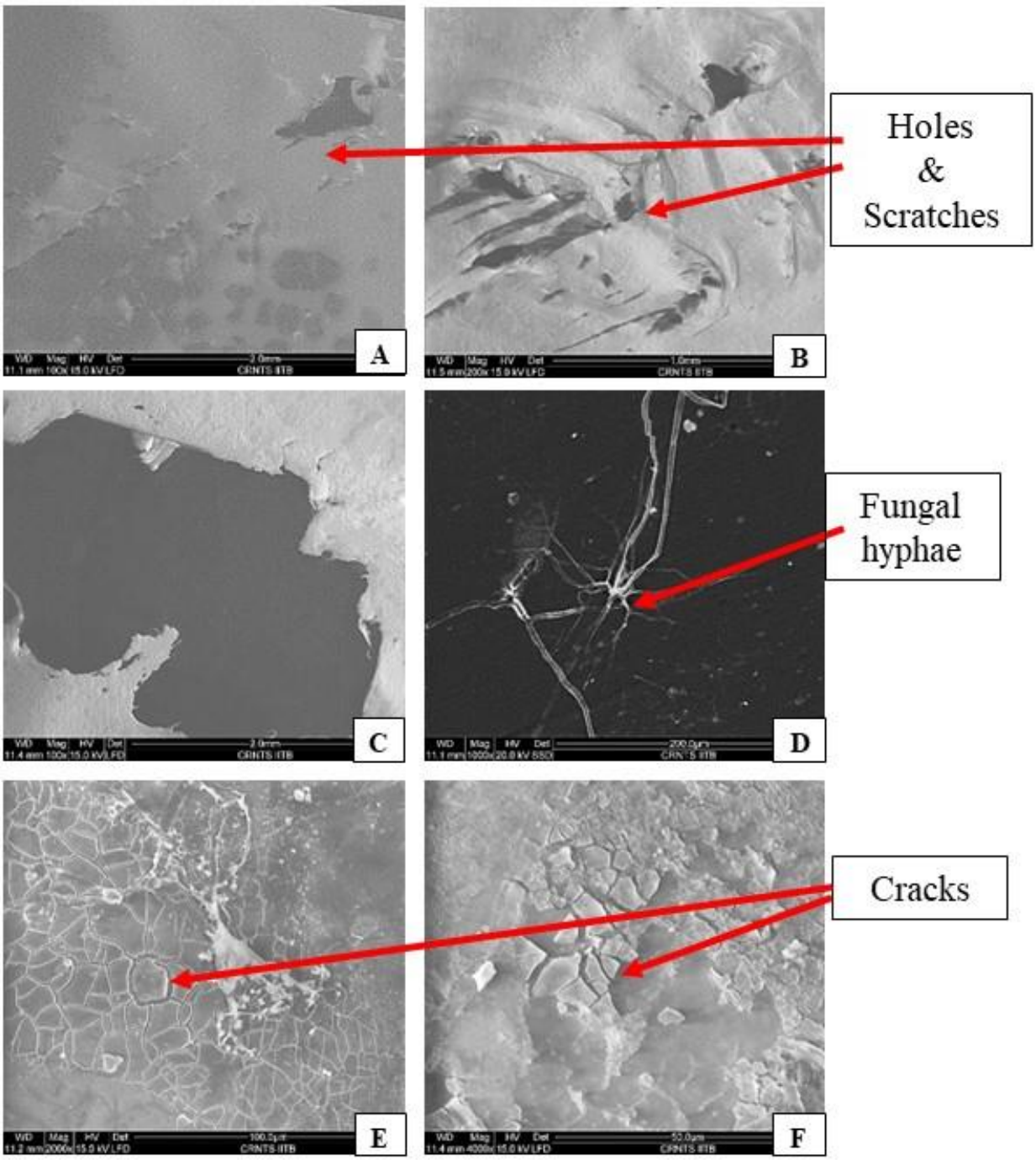


Figure.50. A- Experimented film after 10- & 20-day incubation displaying fungal hyphae penetration, hole and cracks: A & B- Holes and scratches after 10-day incubation (100x, 200x)**; C-** Hole after 20-day incubation (200x)**; D-** Fungal hyphae penetration and cracks on the surface after 10-day incubation(1000x)**; C-** Torn film developing a big hole after 20-day incubation (200x)**; E, F-** Cracks on the surface of experimented PE film (2000x, 4000x)

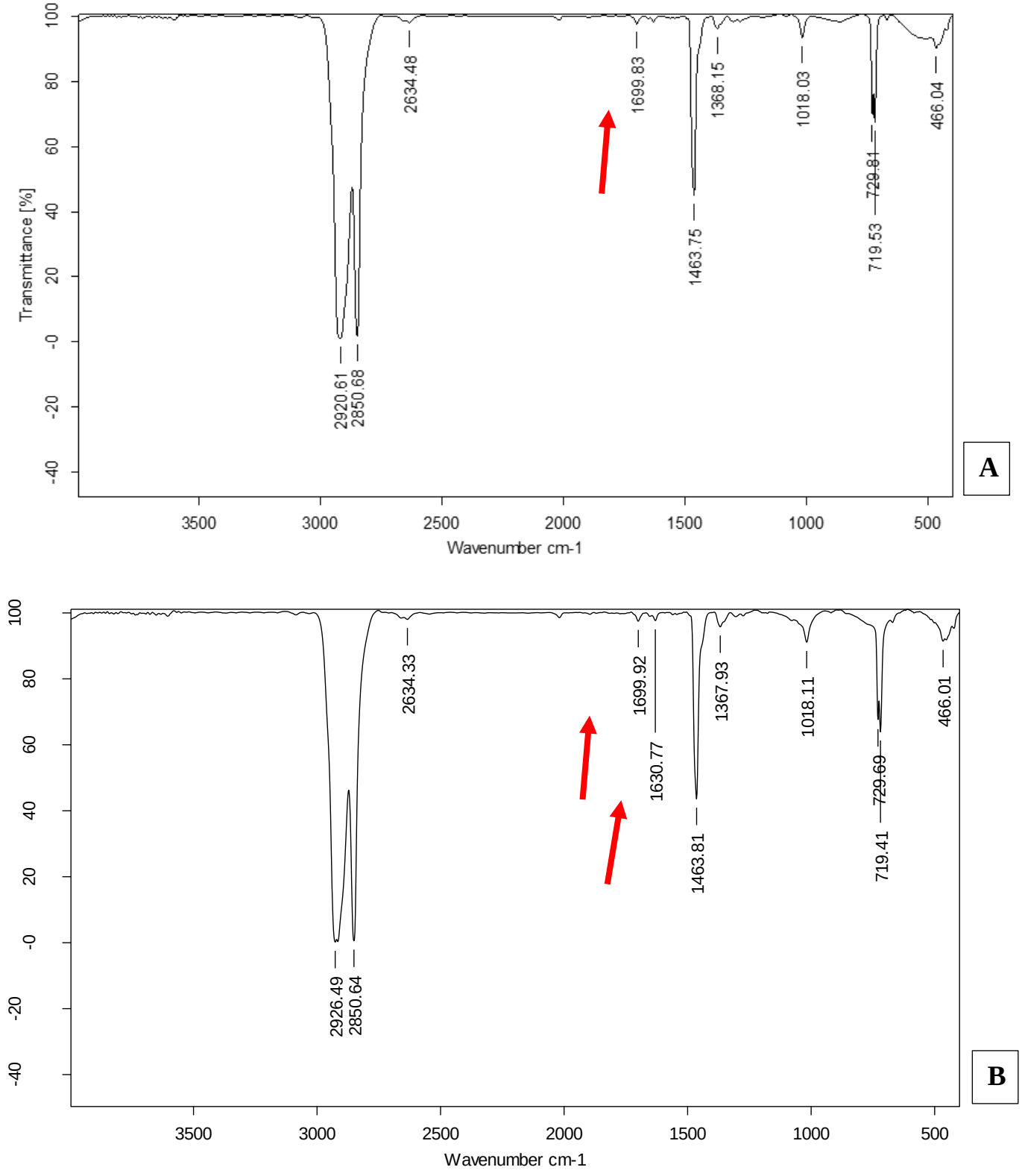


Figure.51. FTIR spectra of experimented PE films in open field area at optimum conditions: **A-** PE film harvested on 10th day**; B-** PE film harvested on 20th day (arrows- formation of new peaks)

Changes in chemical properties (FTIR): FTIR was carried out the films with maximum weight-loss to determine the chemical changes due degradation. The FTIR spectra of film removed after ten

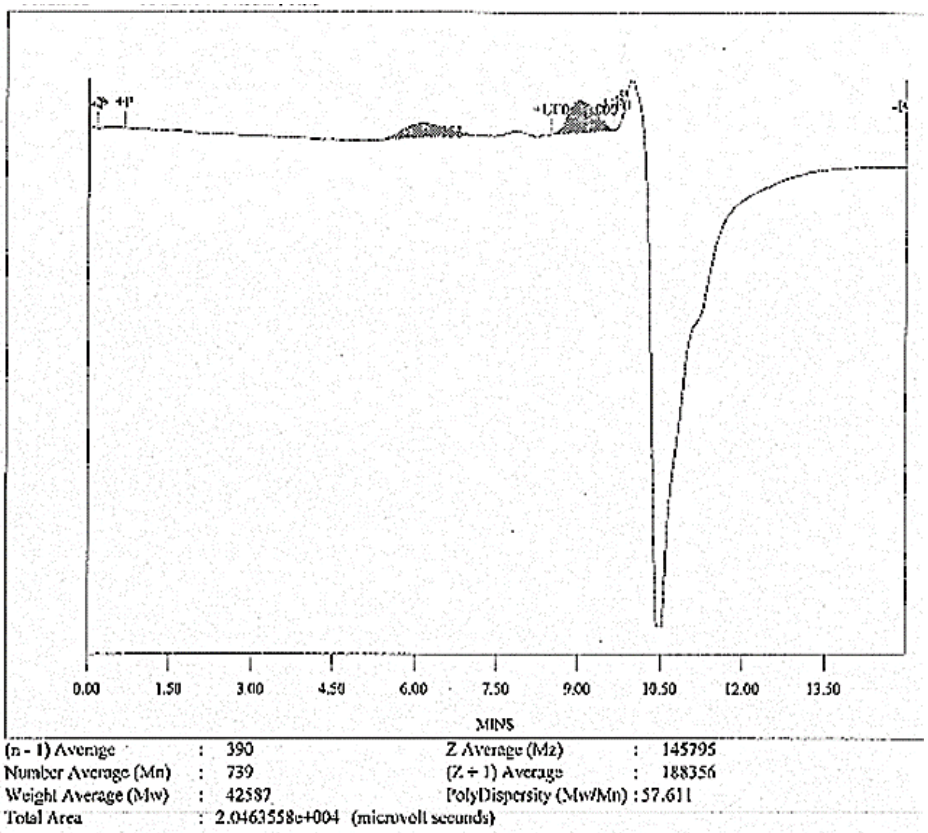
days of incubation showed new peak formation at 1699 cm-1 indicating the formation of carbonyl-containing compounds due to the oxidation by *F. solani* MN201580.1. A peak shift was observed from 2924 cm-1 (Figure.51-A) to a lower wavenumber at 2920 cm-1 (Figure.51-A), which explains that the bond has weakened after 10 days of incubation. FTIR spectra of 20 days incubated film showed additional peak at 1630 cm-1 (Figure.51-B) similar to the one observed in film experimented under lab condition (Figure.51-B). Previous studies have reported new peak formation at 1630 cm-1 and in the range of 1630- 1840 cm-1 after plastic degradation (Konduri et al., 2011; Carduner et al., 1998). These findings provide the confirmation of polyethylene degradation occurred due to *F. solani* MN201580.1 in open field condition.

Changes in molecular weight (Gel permeation chromatography- GPC study): The changes in molecular weight of the film indicates the change in polymeric structure. The degradation stimulates average weight (Mw) and polydispersity (PD), the increase in Mw refers to chain build up and decrease refers to chain breakdown followed by broadened PD (Moss & Zweifel, 1989; Hinsken et al., 1991). The experimented films with higher weight-loss percentage were analysed under GPC to determine the changes in molecular number. The results of GPC analysis showed drastic decrease in Mw, average number (Mn) and PD after pre-treatment and fungal degradation (Figure.52 (A,B) & 53, Table.30). Moreover, GPC data represented elevated and multiple peaks of components after fungal degradation.

Table.30. GPC analysis of control, pre-treated and fungal treated films

Sample	Number Average (Mn)	Weight Average (Mw)	Polydispersity (PD)
Control	739	42587	57.611
Nitric acid treated	470	611	1.300
Fungal treated	124	155	1.247

The untreated film showed 739 Mn, 42587 Mw & 57.611 PD, while these numbers were observed to be reduced after nitric acid treatment. The nitric acid treated film showed 470 average number (Mn), 611 Mw and 1.300 polydispersity. These data were further reduced to 124 Mn, 155 Mw and 1.247 polydispersity after degradation by *F. solani*. The nitric acid treatment has reduced 36% Mn, 98% Mw and 97% PD. The fungal degradation could decrease 73% Mn, 74% Mw and 4% PD in polyethylene film. The incubation of polyethylene with *Acinetobacter baumannii* in synthetic medium for 30 days resulted into 0.62 ± 0.062% weight-loss leading to 9% reduced Mn, 2% reduced Mw and 8% increased PD (Zhang et al., 2022). Gao et al. (2022) reported 95% molecular weight after incubation of polyethylene with a marine fungus *Alternaria alternata* for 120 days. Whereas *F. solani* MN201580.1 showed potentiality to significantly reduce 74% molecular weight of polyethylene film experimented in soil and mulch media for 20 days at open field condition.



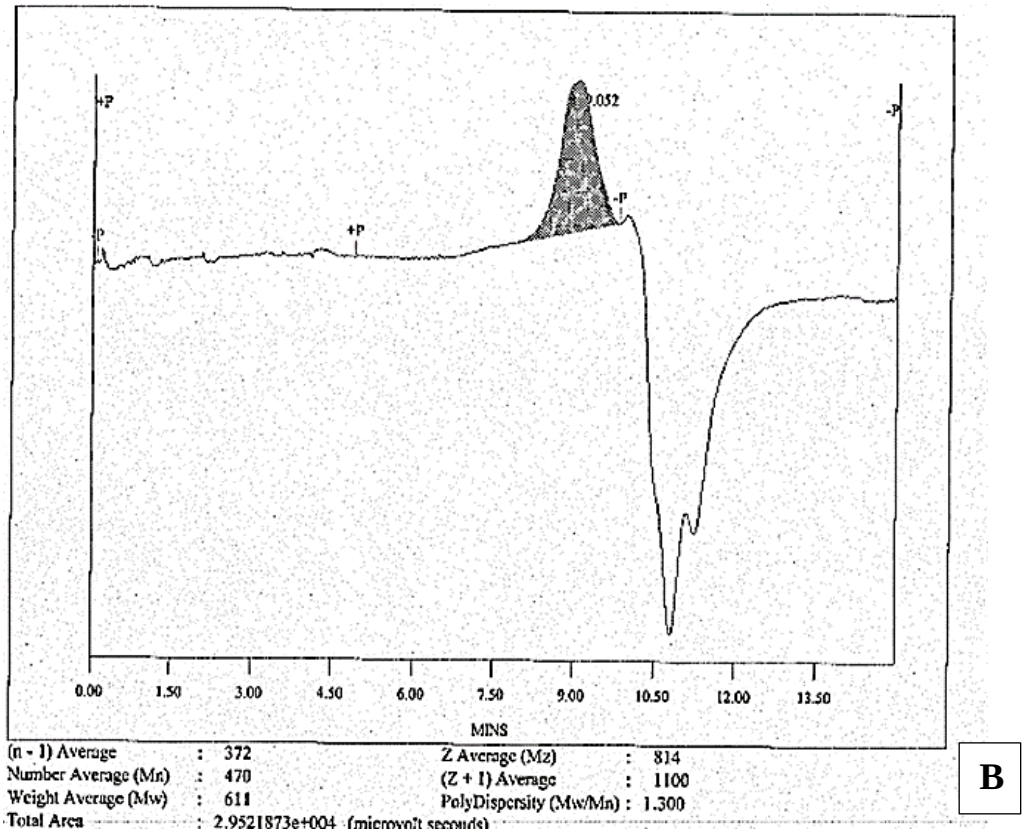


Figure.52. Gel-permeation chromatography analysis: A- Untreated PE film**; B-** Nitric acid pre-treated PE film

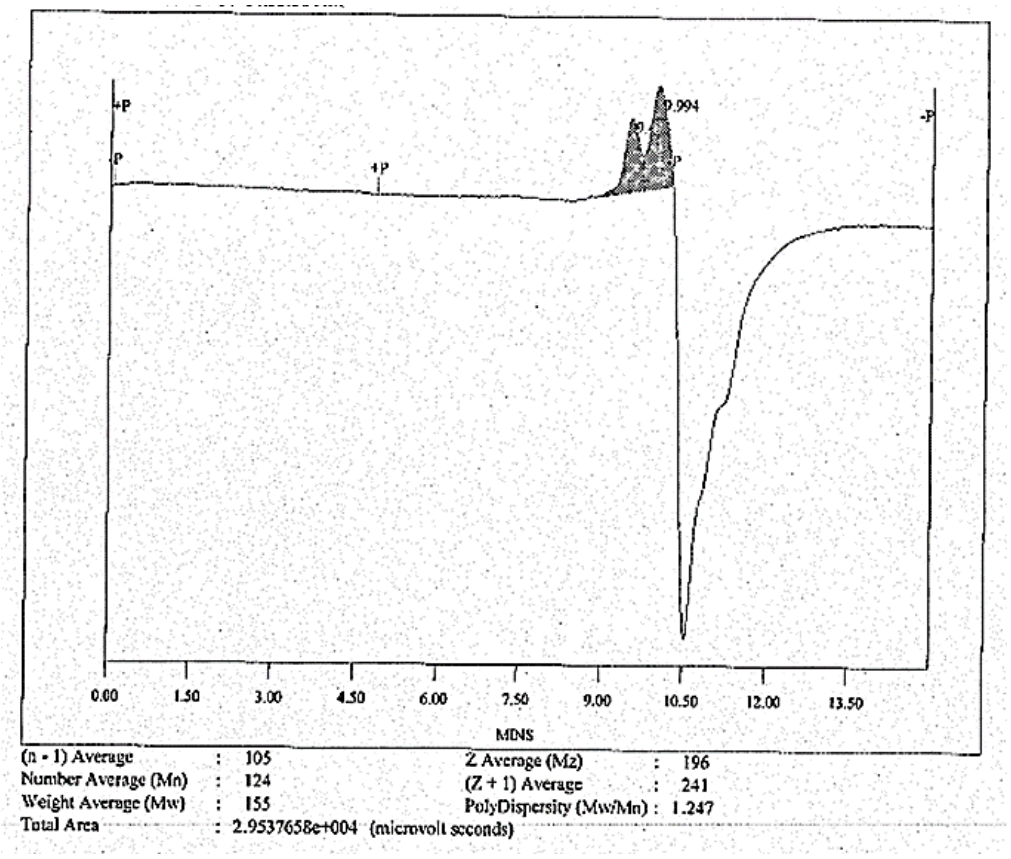


Figure.53. Gel-permeation chromatography analysis: Fungal treated PE film at optimum conditions

Changes in thermal behaviour (Differential Scanning colorimetric analysis- DSC): The changes in melting point and energy peaks can be attributed to variations in the structure of polyethylene. Gulmine et al. (2003) stated that new energy absorption peaks and broadened endotherm graph were observed due to degradation. The DSC analysis of experimented films was performed to determine the variations in thermal behaviour after degradation with *F. solani* MN201580.1. The DSC analysis revealed reduction in melting point, peak height, enthalpy change (Figure.54 and Table.31).

The DSC thermogram of untreated film showed peak temperature at 127.36 °C with 8.16 mW height and area of 292.68 mJ. The sample consumed 109.29 J/g energy between onset temperature 122.63°C to end temperature 129.73°C. The nitric acid pre-treated film showed almost similar peak temperature at 127.63 °C with 8.81 mW height and increased area of peak as 353.66 mJ. It showed increased energy consumption 140.95 J/g between 122.96 °C and 130.32 °C onset & end temperatures, respectively.

The films with pre-treatment and fungal experimented showed reduction in peak temperature and onset temperature with increased peak height. The peak temperature recorded to be 126.87 °C with 9.05 mW and 355.33 peak area, it consumed 142.11 J/g enthalpy for the peak. The onset and end temperatures were recorded to be 121.47 °C and 129.25 °C, respectively. The films experimented

with pre-treatment & fungus along with tween 80 supplementation showed significant variations in thermal behaviour due to degradation. The peak temperature reduced to 126.5 °C with peak height of 6.36 mW and 226.5 mJ area. This sample showed enthalpy drop to 86.6 J/g with onset temperature 121.82 °C & end temperature 128.8 °C.

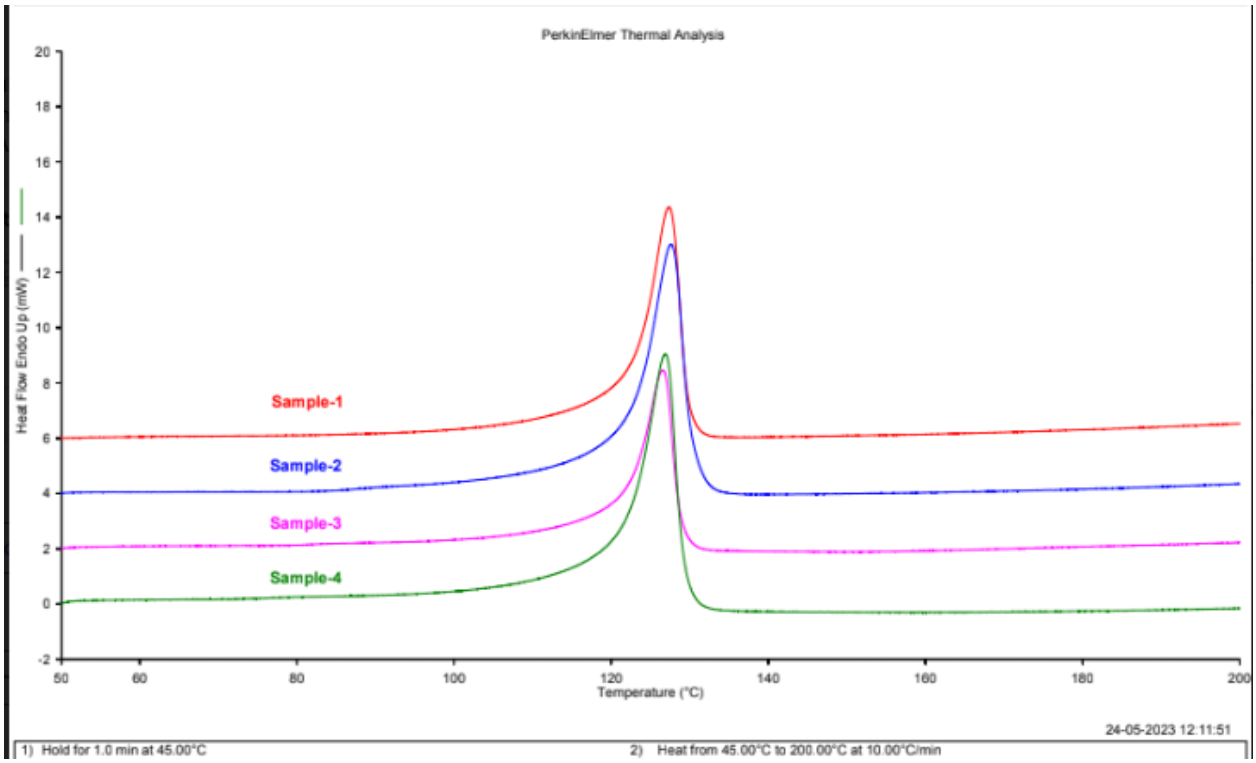


Figure.54. An overlay thermogram of Differential Sanning calorimetry (DSC) analysis performed on experimented films: Sampe 1- Untreated PE film**; Sample 2-** Pretreated film without fungal treatment**; Sample 3-** Pretreated film experimented with SA17 with tween 80 supplementation**; Sample 4-** Pretreated film experimented with SA17 without tween 80 supplementation

Table.31. DSC analysis of experimented polyethylene films

Sample	Peak temperature (°C)	Peak Height (mW)	Area under the peak (mJ)	ΔH (Enthalpy change) (J/g)	On set temperature (°C)	End temperature (°C)
Control	127.36	8.16	292.6836	109.2918	122.63	129.73
Pre-treated	127.63	8.81	352.6602	140.9513	122.96	130.32
Pre-treatment +SA17	126.87	9.0527	355.33	142.1151	121.47	129.25
Pre-treatment +SA17+ tween 80	126.54	6.3619	226.5089	86.6191	121.82	128.83

The higher crystallinity percentage in polyethylene can be attributed to higher degradation rate and amorphous phase of plastic material (Canopoli et al., 2020; Andrady, 2017). The crystallinity percentage of film was calculated as 78% when experimented with *F. solani* MN201580.1 in soil and

mulch supplemented with tween 80. Canopoli et al. (2020) reported 51% crystallinity percentage in 10-year-old aged polyethylene material collected from landfill area. The phenomenon describes the potentiality to break-down polymer and change its properties in 20 days of treatment.

Tensile Strength: The reduced tensile strength of polymer can be referred to the degradation of the material. Tensile strength is a measure to determine the change in mechanical properties of polyethylene due to biodegradation. The reduction in tensile strength of polyethylene indicates fungal degradation. The films were tested for reduction in tensile strength with Universal testing machine (Figure.55) and considerable reduction in tensile strength has been observed after 20-day fungal degradation by *F. solani* MN201580.1.

Table.32. Tensile strength results of experimented polyethylene films

Sample	Tensile strength (MPa)	Elongation at Break (mm)
Control	1.12	98
Pre-treated	1.06	79
Pre-treatment +SA17	1.01	75
Pre-treatment +SA17+ tween 80	0.88	69

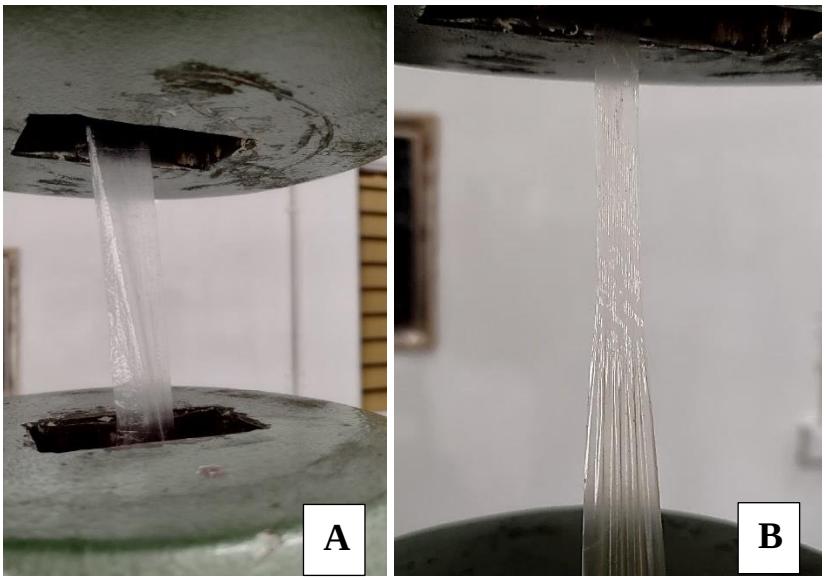


Figure.55. Tensile strength analysis of the experimented PE films: A-PE film at the starting point**; B**- PE film right before the ending point

The control film exhibited 1.12 mPa tensile strength (TS) with 98 mm elongation at break (EAB), which was reduced to 1.06 MPa and 79 mm, respectively, after nitric acid treatment. The biodegradation of polyethyelene by *F. solani* MN201580.1 resulted into reduced tensile strength and elongation at break, it was recorded to be 1.01 MPa and 75 mm, respectively. The supplementation of tween 80 in the media has significantly enhanced the degradation. The sample exhibited 0.88 MPa tensile strength and 69 mm elongation at break when tween 80 was induced. The pre-treatment of

nitric acid showed 5.3% reduced, which was further reduced to 9.82% when treated with *F. solani* MN201580.1.

The pre-treated film experimented for 20 days with *F. solani* MN201580.1 in soil & mulch medium supplemented with tween 80 showed 21.42% reduction in tensile strength. This could be because of the brittleness which may have occurred due to thermo-chemical oxidation and biodegradation. The consortium of bacterial strains isolated from Bengaluru, India showed TS & EAB from 19 ± 3 MPa and 18 ± 3 mm to 16 ± 3 and 13 ± 3 mm, respectively, after 120 days of incubation in synthetic medium. In the present experimental set-up, an enhanced degradation is observed in natural growth medium. Therefore, it clearly depicts that the combination of pre-treatment, *F. solani* MN201580.1 and tween 80 supplementation could significantly change the mechanical properties of the polyethylene.

The degradation of polyethylene by *F. solani* MN201580.1 was analysed by several parameters which revealed substantial degradation in short period of time. Among all kinds of experiment set-up combination of pre-treatment and tween 80 showed that greater the fungal accumulation it results into an increased degradation. The enzyme activity of microorganism plays a vital role in oxidation of the hydrocarbon backbone of polyethylene. *P. chrysosporium* showed potential polyethylene degradation with 18.0 ± 0.5 U/ml MnP activity and 0.5 ± 0.1 U/ml laccase activity in six days (Iiyoshi et al., 1998). In comparison to that *F. solani* MN201580.1 (SA17) exhibited less laccase and MnP activity although it has presented substantial degradation rate. Sivan (2011) stated that molecular weight reduction is one of the key indicators of polyethylene degradation and reported 20% molecular weight and 15% average molecular number of polyethylene when incubated with cell-free laccase. Contradictorily *F. solani* MN201580.1 had potentially reduced 74% average molecular weight and 73% average molecular number after 20 days of incubation in soil and mulch.

The lipase enzyme from *Rhizopus delemer* degraded 53% of the polyester type-polyurethanes (ES-PU) film after 24 hours of incubation (Tokiwa et al., 2009). However, this experiment was conducted in synthetic medium, while in this investigation, *F. solani* MN201580.1 exhibited 41% after 20 days when experimented in soil and mulch. The morphological changes observed under SEM revealed cracks in mosaic pattern, fungal hyphae penetration and deposition of presumably fragmented polymer on the film, are some of the features which bears resemblance to observations made in the study conducted by Gulmine et al. (2023) on the weathering degradation of polyethylene. Furthermore, FTIR analysis showed formation of new peaks and peak shifts after degradation providing the evidence of polyethylene breakdown. The changes in thermal behaviours like reduced melting point and increased crystallinity can be attributed to the degradation of polymer. The present

study has evidentially observed these changes in thermal properties of polyethene after its degradation by *F. solani* MN201580.1.

Nwuzor et al. (2023) showed addition of 50 wt.% of plasticized cassava starch (PCS) to the LDPE blend reduced the tensile strength from 15.08 – 5.25 MPa, and elongation at break from 18.93 – 9.32 mm. The study suggested new composition of polyethylene optimal environmental pollution control. The present study recorded a reduction in tensile strength from 1.12 to 0.88 MPa and elongation at break 98 mm to 69 mm, which could be an distinctive solution to the already existing polyethylene waste.

On the whole, the potentiality of *F. solani* MN201580.1 (SA17) to breakdown polyethylene backbone in open-field condition has been evaluated and demonstrated by analysing physical, mechanical, chemical, thermal and molecular characteristics.

Key observations of the study

The degradation of polyethylene was evaluated at lab-simulated and open-field simulated condition by keeping parameters at optimal level.

- Soil and mulch medium similar in composition to be open field was found to be the best substrate for fungal biodegradation of polyethylene which is pretreated with nitric acid and enhancer tween 80.
- All five enzyme activities were optimum after fifteen and twentieth days of incubation in soil and mulch medium.
- The maximum weight loss was recorded after 20 days with 40 ± 0.14% and 41.5 ± 0.56% in lab-stimulated and open-field experiment, respectively.
- The topographic changes like cracks and holes due to fungal hyphae penetration was observed in SEM analysis.
- FTIR analysis revealed new peak formations and peak shifts and depicted changes in chemical composition due to fungal degradation.
- The average molecular weight and average molecular number were significantly reduced to 74% and 73%, respectively.
- The melting point of experimented film was recorded to be reduced and crystallinity of the film was increased to 78% due to degradation.
- Tensile strength of the polyethylene film was reduced to 21.42% after 20 days of incubation.

To conclude *F. solani* MN201580.1 showed its potentiality to degrade polyethylene film significantly only after pre-treatment with nitric acid followed by a substrate of soil and mulch supplemented with tween80 as an enhancer for the degradation within 20 days of incubation.

Without nitric acid pretreatment the degradation was negligible. Without tween 80, degradation was 21.15% in 20 days while when supplemented with tween 80 the degradation increased by 20% (41.5% weight loss in 20 days).