CHAPTER 5

Fungal ligninolytic, lipolytic, and proteolytic enzymes were quantified and optimized in previous experiments. Fungal strain *F. solani* MN201580.1 (SA17) showed a significant amount of enzyme secretion on the 20th day of incubation with a single disc inoculum in a culture medium at pH 8 and 30ºC temperature. The potential to degrade a substantial amount of polyethylene was identified in culture strain SA17. However, in terms of practical application for bioremediation, enhancing the degradation is essential to manage the waste. The optimal parameters were set for further experimentation of enhancement. In this investigation, the selected fungal strain *F. solani* MN201580.1 (SA17) was chemically and biologically tested for enhanced degradation capability.

Enhancement of plastic degradation under optimized condition using different enhancers. (Chemical & Biological)

The enhancement experiment was conducted in two parts: biotic augmentation (Fungal consortium) and abiotic augmentation (Chemical). Biotic and abiotic augmentation aids in enhancing the capability of fungal strains to breakdown complex polymers (Dsouza et al., 2021; Ghatge et al., 2020). In the biotic augmentation experiment, compatible fungal strains were identified through a paired interaction test. The objective was to find among the potential plastic degrading strains that could interact effectively with *F. solani* MN201580.1 (SA17) and enhancing the degradation potential. Once compatible strains were identified, they were grown together to test their collective capability to degrade polyethylene material. This biotic approach aimed to evaluate the synergistic effects of combining *F. solani* MN201580.1 (SA17) with other strains to enhance the overall polyethylene degradation process. For the chemical augmentation, *F. solani* MN201580.1 (SA17) was evaluated for an enhanced degradation capacity by adding chemical enhancers (Mineral oil, Tween80, Soluble Starch) to the soil and mulch growth medium. The aim was to assess the impact of these chemical enhancers on *F. solani* MN201580.1 (SA17)'s ability to degrade polyethylene.

METHODOLOGY

Preparation of culture replicates in solid state medium for the degradation

The polyethylene films were aseptically placed into 1 kg of soil and mulch mixture containing dry kitchen waste procured from Concept Biotech, Vadodara. The mulch consisting kitchen waste and garden waste mainly vegetable peels, rotten fruits, egg shells and dried leaves was used for the experiment. Each replicate was then inoculated with a 9mm fungal disc and incubated for six different periods: 2, 4, 6, 8, 12, and 16 weeks. A total of 1 gm of polyethylene strips were layered with soil and mulch, and on each layer, five 9mm inoculum discs were positioned. Control experiments were also conducted, which involved only the polyethylene films placed in the growth medium without any

fungal inoculum. At the end of each incubation period, the replicates were harvested, and the polyethylene films were washed and dried.

(A) Biotic augmentation:

In the biotic augmentation approach, a co-culturing technique was employed to assess the potential of different consortia composed of compatible co-partners to enhance polyethylene degradation. The compatibility test was conducted to determine the suitability and interaction between various strains. The strains evaluated for compatibility were *A. oryzae* STR1 (SA5), *A. oryzae* STR2 (SA15), *F. solani* MN201580.1 (SA17), and *A. tubingensis* (SA1). Further potential fungal consortium was identified by the degradation experiment.

Compatibility Test

A paired interaction test was performed to check compatibility of all four potential fungal strains with each other. A 9 mm inoculum discs of two different strains were inoculated in a petri-plate and at different time intervals fungal growth was observed. A paired interaction of fungi could be considered once they come in contact and still each one grows over the other at its own pace with the formation of an overlapping zone which increases towards both the sides and called as mutual intermingling. When one fast growing fungus grows over & kills the other it depicts negative phenomena for compatibility test and called as invasion as explained by Porter (1924). There are four types of interactions as mentioned below:

1. Mutually intermingling: Where both fungi grew into one another without any macroscopic signs of interactions.
2. Intermingling growth: (i) where the fungus grew into the opposed fungus either above or below or above and below its colony, and its corollary. (ii) Where the fungus under observation has ceased growth and is being overgrowth by another colony.
3. Slight inhibition: Where the fungal growth approach each other until almost in contact and a narrow demarcation line, 1-2mm, between the two colonies is clearly visible.
4. Mutual inhibition: A demarcation line at a distance of >2mm between the two colonies.

Identification of potential consortia

To identify potential consortia with enhanced polyethylene degradation activity, compatible strains were grown together as consortium and their effectiveness was evaluated. The experiment involved using pre-treated polyethylene (PE) films, which were removed at different intervals (2, 4, and 6 weeks) to analyze the weight loss as an indicator of degradation. The weight loss of the PE films served as an indication of the efficiency of each consortium in degrading the polyethylene material. Three different consortiums were experimented with, each containing of *F. solani* MN201580.1 with other potential fungal isolates.

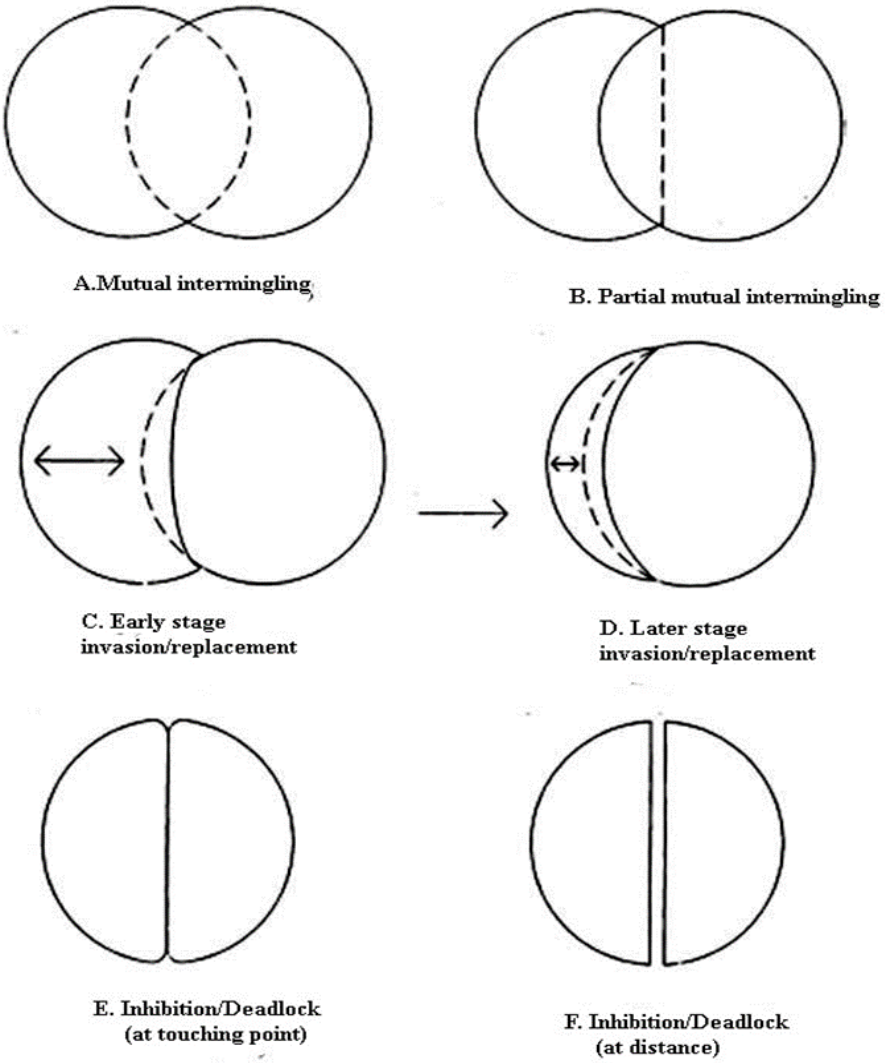

Figure.37. Schematic diagram showing interactions between two different fungal isolates grown adjacently (4cm apart) on medium observed after one week incubation period (based on the observations of Porter (1924)

(B) Abiotic augmentation:

In this set up, three pre-treatments were performed on PE films (Meinel, G., & Peterlin, A. 1968; Nakae et al., 2000; Nwachukwu et al., 2010):

(i) 60°C + Concentrated HNO_3: The samples were treated with heat at 60°C, followed by exposure to concentrated nitric acid; (ii) 60°C + 0.5 N HNO3: The samples were treated with heat at 60°C, followed by exposure to 0.5 N nitric acid; (iii) 60°C + 0.5 N NaOH: The samples were treated with heat at 60°C, followed by exposure to 0.5 N sodium hydroxide (NaOH). Treated samples were experimented in mixture of soil + mulch media along with the enhancers and 9mm fungal inoculum disc as previously explained.

To investigate the impact of various known chemical enhancers on polyethylene degradation, mineral oil, Tween 80, and soluble starch were utilized. Each enhancer was supplemented separately at a concentration of 0.05% along with the polyethylene (PE) film and fungal inoculum into the

Soil+Mulch medium (Tribedi & Sil, 2013; Hadar & Sivan, 2014). The effects of these enhancers were assessed by analyzing the PE films for weight-loss, scanning electron microscopy (SEM), and Fourier-transform infrared spectroscopy (FTIR). These analyses aimed to determine the extent of polyethylene degradation and to observe any changes in the film's structure and chemical composition resulting from the presence of the enhancers.

RESULT AND DISCUSSION

Enhancing fungal degradation is essential to scale up the practical application. For this purpose, the experiment was set up in two ways: abiotic and biotic augmentation. An approach of abiotic augmentation approach involves adding chemical enhancers that increase fungal accumulation on the PE film, resulting in higher degradation. Biotic augmentation approach uses a co-culture technique, which induces competitional stress and leads to higher enzyme secretion, ultimately increasing the percentage of weight-loss.

(A) Biotic augmentation: In this experiment, the process of polyethylene degradation was tested for the enhancement by identifying the potential fungal consortia. To identify the suitable consortia, all potential fungal strains were cocultured to study the different interactions and identify their compatibility to grow.

Paired interaction test

In the screening of polyethylene degradation, out of the nineteen strains tested, *A. tubingensis* (SA1), *Pestalotiopsis* sp. (SA13), *F. solani* MN201580.1 (SA17), *A. oryzae* STR1, and STR2 (SA5 & SA15) showed significant results in terms of their potential to degrade polyethylene. Microbial consortiums are known to work in a multidisciplinary way on complex structures, breaking them down into simpler segments (Sowmya et al., 2015). Thus, to accelerate the degradation process, co-culturing these strains was considered. Before experimenting with the consortium of these strains and polyethylene films, a paired interaction test was conducted to determine their compatibility.

Various interactions of the five fungal isolates were studied for two incubation periods, three and six days. Among all the fungal strains, the growth of *A. oryzae* strains was the fastest, and *F. solani* MN201580.1 (SA17) also exhibited rapid growth. Both fungal strains *A. oryzae* STR1 and STR2 (SA5, SA15) showed compatibility with *Pestalotiopsis* sp. (SA13) and *F. solani* MN201580.1 (SA17) isolates, as observed in Figure.37 and Table.23.

- Both fungi came in contact and growth of both fungal isolates were inhibited i.e. No further growth occurred once the two came in contact.
- The growth of one was inhibited by the other but it was not killed. The fungal isolate grew on the counterpart.
- Both strains came in contact; one overgrew over the other and killed it.

Literature survey indicates co-culture technique to be beneficial for enhancement of enzymatic activities (Mewada et al., 2017). Here in all ten paired interactions, only four types of mutual intermingling were observed, where both fungi grew well without killing each other. Two types of partially mutual intermingling were observed, which occurred when both fungi grew equally without killing each other. Total four types of combinations showed incompatibility between the strains.

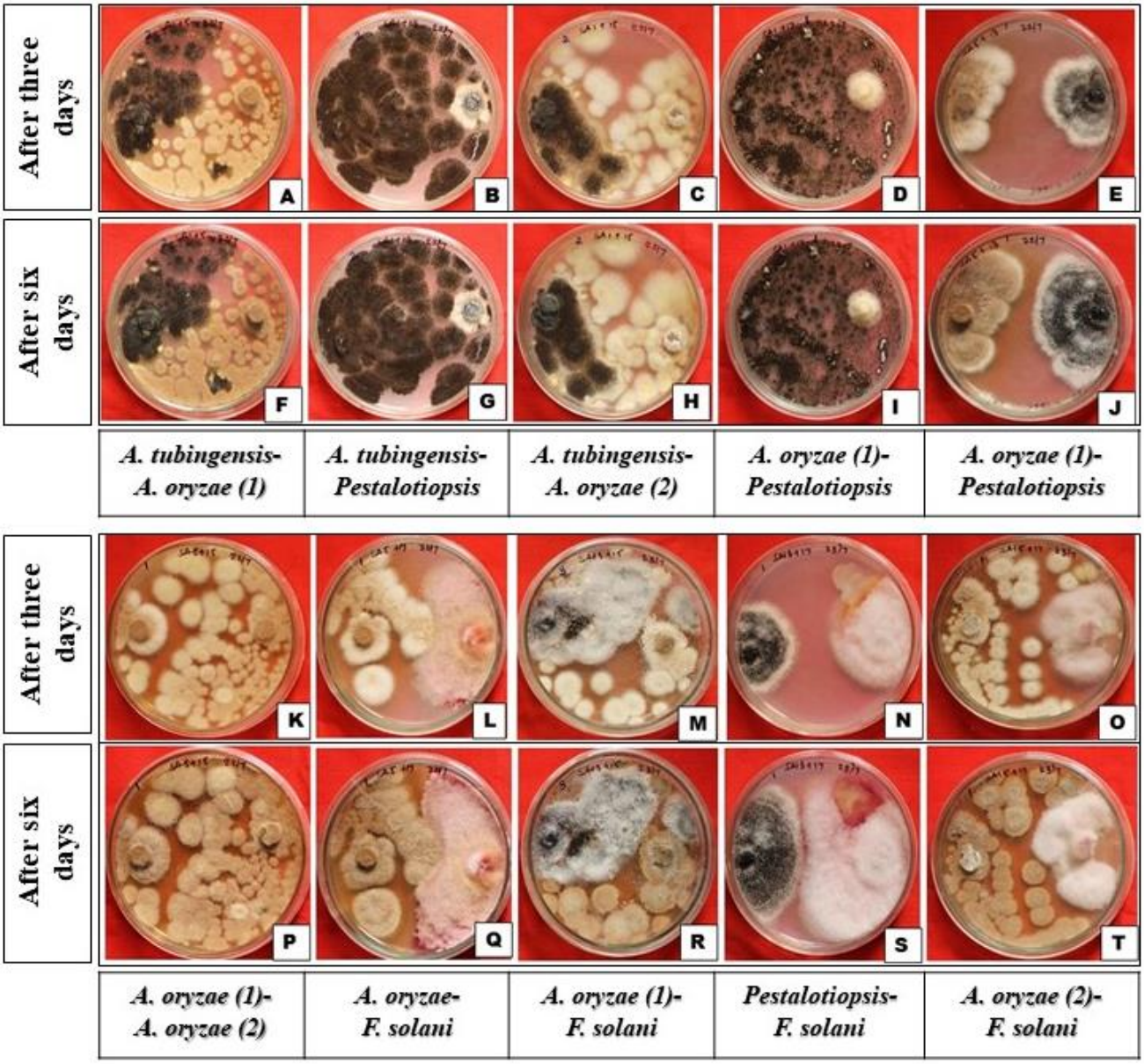

Figure.38. Paired interaction test of five fungal strains:
A & F- SA1 and SA5; **B & G**- SA1 and SA13; **C & H**- SA1 and SA15; **D & I**- SA1 and SA17; **E & J**- SA5 and SA13; **K & P**- SA5 and SA15; **L & Q**- SA5 and SA17; **M & R**- SA13 and SA15; **N & S**- SA13 and SA17; O & T- SA15 and SA1

In the ten interactions of different fungal isolates, several patterns of compatibility and growth rates were observed. The growth of *A. oryzae* strains (especially *A. oryzae* SA5) was very fast compared to other fungal isolates. *A. oryzae* SA15 showed compatibility with all other fungal isolates, indicating

its potential to coexist and grow together with different strains. *A. tubingensis* (SA1) did not show compatibility with most strains except *A. oryzae* (SA5). It also showed compatibility with *A. oryzae* (SA15). *A. tubingensis* (SA1) restricted the growth of *F. solani* MN201580.1 (SA17) and *Pestalotiopsis* sp., leading to inhibition or antagonistic interactions with these strains. *Pestalotiopsis* sp. showed compatibility with both strains of *A. oryzae* but had restricted growth when interacting with *F. solani* MN201580.1 (SA17). *F. solani* MN201580.1 (SA17) exhibited compatibility with both strains of *A. oryzae*, indicating that it can coexist and grow together with these strains.

Table.23. Characteristic features observed on 3rd and 6th day of incubation of different fungal isolates grown together

Name of fungi	3rd day	6th day	Nature of interaction
SA1-SA5 Fig.38 (A&F)	Fungal growth of SA1 was faster than SA5, both the fungi were grown equally on the 3rd day after inoculation. At the same day both fungi came in to contact with each other.	The situation remained same further, and showed deadlock type of interaction at the distance. Both fungal growths inhibited by each other	Inhibition/Deadlock (At distance)
SA1-SA13 Fig.38 (B&G)	In the paired interaction between SA1 and SA13, SA1 exhibited rapid growth, almost covering the entire petri-plate by the 3rd day after inoculation. On the 3rd day, sporulation of SA1 had just started, and it continued to grow and overgrow till the inoculum disc of SA13.	The growth of SA13 was inhibited by SA1. This indicates that SA1 acted as an antagonist against SA13, leading to an early-stage invasion or replacement interaction.	Early-stage Invasion/ replacement
SA1-SA15 Fig.38 (C&H)	SA1 and SA5 both strains grew well and came into contact with each other on 3rd day of inoculation, however SA5 grew faster than SA1.	On sixth day, both the fungi came in to contact with each other and growth of SA15 continued till 15 days. These two fungi are compatible with each other even the growth of SA5 was very rapid and this type of interaction was found to be partial mutual intermingling.	Partial Mutual Intermingling
SA1-SA17 Fig.38 (D&I)	Fungal growth of SA1 was faster than SA17. Sporulation of SA1 started and covered the whole plate on the 3rd day of incubation, and growth of SA17 was just started.	After six days of incubation, growth of SA17 was inhibited by SA1 and SA1 overgrew on SA17. SA1 strain acted as antagonist against SA17 and this interaction was found to be early-stage invasion/replacement.	Early-stage Invasion/ replacement
SA5-SA13 Fig.38 (E&J)	Both fungi SA5 and SA13 grew equally and well on third day after inoculation.	After six days, both the fungi came in to contact with each other, and SA5 growth was continued till 15 days after inoculation. Growth of SA5 continued till 15 days. Hence these two fungi are partially compatible with each other and displayed partially mutual intermingling.	Partial Mutual Intermingling

SA5-SA15 Fig.38 (K&P)	In this interaction both fungal strains SA5 & 15 grew evenly and came in contact with each other on third day of incubation.	After six days, both fungi started growing on each other as these strains belong to the same genera. Therefore, these two strains are compatible with each other and exhibited mutual intermingling type of interaction.	Mutual Intermingling
SA5-SA17 Fig.38 (L-Q)	SA5 and SA17 both fungi grew equally and came into the contact with each other on third day of inoculation.	These two strains did not inhibit each other's growth and started overgrowing on each other on sixth day. This situation continued even after fourteen days, hence this interaction exhibited mutual intermingling.	Mutual Intermingling
SA13-SA15 Fig.38 (M-R)	During this interaction, SA13 and SA15 both grew well, however, sporulation of fungi was not occurred on third day after inoculation.	After six days, sporulation of both the fungi started and came into contact with each other. These two fungi grew well in them on pace without harming each other's growth, hence showing the mutual intermingling type of interaction.	Mutual Intermingling
SA13-SA17 Fig.38 (N-S)	The growth of SA17 was faster than SA13 till third day in this interaction.	On sixth day after inoculation, both fungi came into the contact with each other and eventually growth of fungi was stopped. The interaction was found to be invasion/replacement at touching point as the growth was restricted.	Inhibition/Deadlock (At touching point)
SA15-SA17 Fig.38 (O-T)	Here SA15 grew faster than SA17 on third day after inoculation and sporulation started after five days.	After six days, SA17 started growing over the SA15 fungal colonies and the situation continued even after ninth day. This interaction was found to be mutual intermingling and indicated their compatibility with each other.	Mutual Intermingling

Screening of potential consortia

The screening of potential consortia involving co-culture and combinations of fungal strains is an essential step in finding efficient biodegradation solutions for pollutants like polyethylene. Previous studies have shown that fungal consortium can be more effective in pollutant breakdown compared to using single fungal species (D'Souza et al., 2021). The most potential strain *F. solani* MN201580.1 (SA17) was experimented with other four potential fungal strains in different combinations to enhance the degradation rate.

In this investigation, based on the results of the paired interaction test, thirteen combinations of compatible fungal strains were selected to identify the potential consortia for enhancing the degradation of pre-heated (70°C) polyethylene films. Compatible and non-compatible both combinations were tested in the experiment to check their capability to enhance the degradation. By selecting the right combinations of fungal strains, the goal is to create a consortium that can work synergistically to enhance the degradation process and achieve higher rates of polyethylene breakdown.

Co-culture of five fungal strains *A. tubingensis*, *A. oryzae* (SA5 & SA15), *Pestalotiopsis* sp., and *F. solani* was experimented with ten combinations. Among them six combinations were compatible and the rest were not compatible with each other. Although strains were compatible with each other, the combinations failed to enhance the degradation rate and showed negligible findings. Five combinations of compatible partners namely SA1 + SA15, SA5 + SA13, SA5 + SA15, SA5 + SA17, SA15 + SA13, and SA15 + SA17 were experimented and maximum weight loss was achieved after six weeks of incubation (Figure.37 and Table.24). The co-cultures of fungi showed percentage weight-loss ranging from 0- 0.65% during the experiment. In the co-culture experiment of *A. oryzae* (SA15) and *F. solani* MN201580.1 (SA17), even though they exhibited mutual intermingling interaction and compatibility, they did not show a substantial weight-loss percentage when inoculated together. No weight loss was recorded in untreated films, and after two, four, and six weeks of incubation, weight-loss percentages of 1.33 ± 0.002%, 0.84 ± 0.001%, and 0.65 ± 0.002%, respectively, were noted.

Table.24. Percentage weight-loss in experimented PE films in fungal consortium

Combination of fungal strains		**Untreated**			**70° Heat**		
		2weeks	**4weeks**	**6weeks**	**2weeks**	**4weeks**	**6weeks**
Compatible Strains	SA1 + SA15	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.023± 0	0.045 ± 0.002
	SA5 + SA13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.001	0.03 ± 0.02
	SA5 + SA15	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.0001	0.1 ± 0	0.41 ± 0.0003
	SA5 + SA17	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.0001	0.32 ± 0.0002	0.59 ± 0.001
	SA15 + SA13	0 ± 0	0.01 ± 0	0.01 ± 0	0 ± 0	0.22 ± 0.001	0.45 ± 0.02
	SA 15+17	0 ± 0	0 ± 0	0 ± 0	1.33 ± 0.002	0.84 ± 0.001	0.65 ± 0.002
Non-compatible strains	SA1 + SA5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	SA13 + SA17	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	SA1 + SA17	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	SA1 + SA13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Consortia	SA 5+15+17	0 ± 0	0.4 ± 0.0001	0 ± 0	0 ± 0	0 ± 0	0.54 ± 0.003
	SA 5+15+17+1	0.41 ± 0.01	0.27 ± 0.002	1.26 ± 0.003	0 ± 0	3.42 ± 0.004	**4.1 ± 0.005**
	SA1 + SA5 + SA13 + SA15+ SA17	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.29 ± 0.001	0.51 ± 0.002

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

The combinations of non-compatible strains SA1 + SA5, SA13 + SA17, SA1 + SA17, and SA1 + SA13 were also experimented to determine the effect of stress of survival on polyethylene

degradation. However, these combinations did not show any significant weight loss throughout the experiment. The percentage weight loss was recorded to be zero in all four combinations of non-compatible cultures. It concludes that the non-compatible cultures cannot degrade any amount of polyethylene material when cultured together.

The findings of the coculture technique were not significant when compared to the monoculture technique in the previous experiment (Chapter 2). The single culture of fungal strains SA1, SA5, SA13, SA15, and SA17 exhibited 2.63 ± 0.02%, 6.76 ± 0.02%, 3.65 ± 0.01%, 2.57 ± 0.03%, and 17.93 ± 0.05% in four weeks, respectively (Table.11). Where in co-culture techniques the results were very less compared to the monocultures. These results indicate the potentiality of these strains to degrade polyethylene as a single culture.

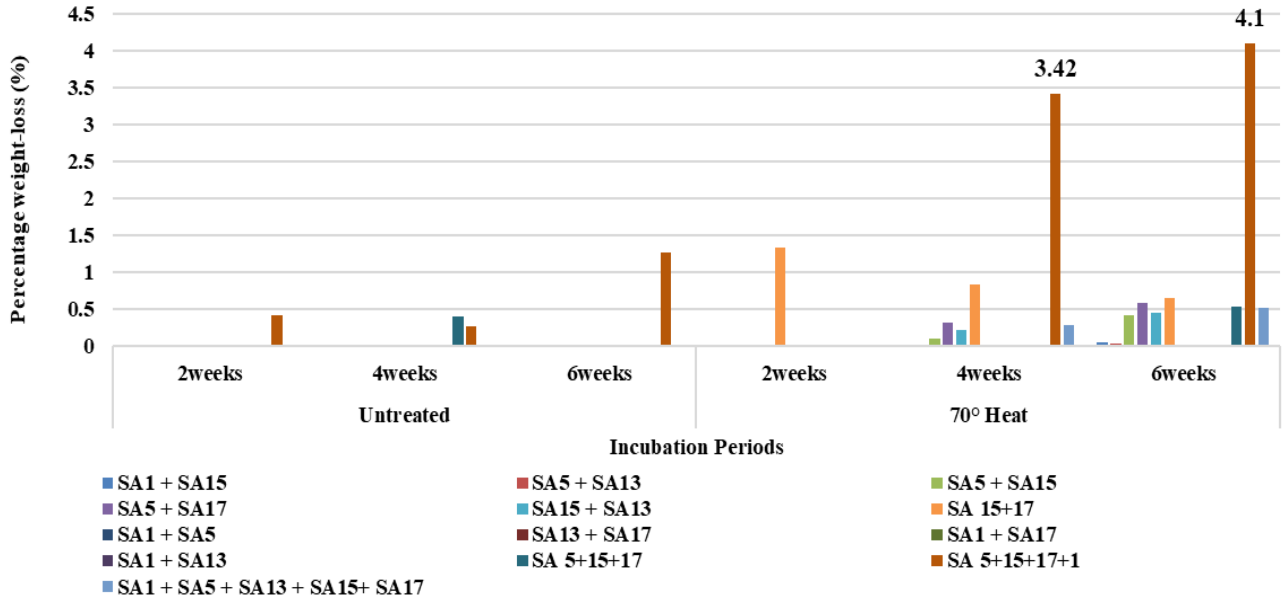

Figure.39. Percentage weight-loss of polyethylene films experimented by co-culture technique

In this experiment, the highest weight-loss was observed after six weeks of incubation using a combination of fungal strains SA5, SA15, SA17, and SA1. This consortium achieved a degradation rate of 4.1% for the 70°C pre-heated polyethylene film (Figure.37 & Table.24). As in previous experiments, the pre-treatment of PE films stimulated the degradation process by the fungi. Despite the compatibility of these strains with each other, the consortium experiment did not achieve a significant percentage weight-loss compared to the previous experiments.

The tri-culture of *A. oryzae* (SA5), *A. oryzae* (SA15), and *F. solani* MN201580.1 (SA17) (SA5+SA15+SA17) did not show a significant improvement in the degradation process. The data obtained from these tri-culture replicates were negligible compared to the results obtained from the monoculture replicates of these strains, which exhibited higher weight-loss percentages. For untreated polyethylene films, the combination of SA5+SA15+SA17 represented only 0-0.4% weight-loss,

whereas the single cultures of SA5, SA15, and SA17 achieved higher weight-loss percentages of 1.7 ± 0.01%, 2.78 ± 0.002%, and 4.22 ± 0.01%, respectively, as shown in chapter.2 (Table.11). In heat-treated films, the tri-culture of SA5+SA15+SA17 achieved only 0.54 ± 0.003% weight-loss after six weeks of incubation.

The consortia of all five fungi SA1+ SA5+ SA13+ SA15+ SA17 was experimented with for enhancement which failed to degrade the polyethylene film. The maximum loss was recorded to be 0.29 ± 0.001% and 0.51 ± 0.002% after four and six weeks, respectively, which indicates the incapability of the consortia. On the other hand, the single-culture of SA5, SA15, and SA17 achieved much higher weight-loss percentages of 6.76 ± 0.02% (4 weeks), 9.95 ± 0.002% (2 weeks), and 17.17 ± 0.02% (maximum loss) after incubation.

In this experiment, the consortium of *A. oryzae* (SA5), *A. oryzae* (SA15), *F. solani* MN201580.1 (SA17), and *A. tubingensis* (SA1) (SA5+SA15+SA17+SA1) showed the maximum weight-loss in 70°C heat-treated films. After four weeks of incubation, the weight-loss was recorded as 3.42 ± 0.004%, and it further increased to 4.1 ± 0.005% after six weeks. For untreated films, the weight-loss percentages were lower, with 0.41 ± 0.01%, 0.27 ± 0.002%, and 1.26 ± 0.003% recorded in 2, 4, and 6 weeks of incubation periods, respectively. The data obtained from this consortium experiment was not significant, and as a result, enzyme activity, SEM and FTIR analysis were not conducted for the films harvested during this particular set-up. It is worth noting that when these fungal strains were used individually in monocultures, they presented higher weight-loss percentages, as discussed earlier. However, in the consortium, their degradation capabilities seem to be affected, resulting in lower weight-loss percentages.

The previous reported works on consortia of different fungal and bacterial strains showed significant weight-loss percentages in the degradation of polyethylene. For example, a consortium of *A. niger* and *A. flavus* resulted in 15.625% weight-loss in LDPE strips after four weeks, while a consortium of bacterial strains achieved 21.70% weight-loss in a study by Satlewal et al. Similarly, a consortium of *Curvularia lunata*, *Alternaria alternata*, *Penicillium simplicissimum*, and *Fusarium* sp. demonstrated 27% weight-loss after three months.

However, in this current investigation, the combination of fungal strains in consortia did not result in a substantial increase in weight-loss percentage compared to single culture techniques. This suggests that the potential of these fungal strains to degrade treated polyethylene film might be more effective when used individually rather than in combination. The variation in results between different studies could be attributed to specific strains and their compatibility between each other.

In conclusion, the abiotic augmentation using chemical inducer tween 80 resulted in significantly higher weight-loss percentages compared to the biotic augmentation using fungal consortia. The

maximum weight-loss achieved in the tween 80 induced culture replicates of *F. solani* MN201580.1 (SA17) was 34.7% higher than the consortium of SA5+SA15+SA17+SA1 (maximum loss).

To effectively manage plastic waste using this protocol, it is essential to accelerate the process of degradation by fungi. The experiment was conducted in a natural growth medium of soil and mulch to assess its practical applicability. Based on the results as the monoculture technique showed significant result compared to coculture.

(B) Abiotic augmentation:

The degradation activity could be increased in a shorter period of time by adding enhancers such as 0.05% mineral oil, Tween 80, and soluble starch to the media. In the previous experiment, thermal and chemical pretreatments enhanced the degradation rate. Therefore, thermo-chemical pretreatments were performed on the PE films, and fungal strains were inoculated along with chemical enhancers and kept for the desired incubation periods. Chemical agents like Tween 80 and mineral oil have been reported to stimulate the hydrophobic interaction between polyethylene material and microorganisms, ultimately influencing the degradation of the polymer (Tribedi et al., 2013).

The 60°C heat treatment followed by concentrated nitric acid treatment proved to be effective for the degradation process. According to Chaudhary et al. (2022), acid pre-treatments alter the polymer's composition into a more oxidized form and lower the molecular weight. The nitric acid treated LDPE films achieved 13% weight-loss by *Cephalosporium* species in eight weeks (Chaudhary et al., 2022). In contrast, in this investigation, a 38.8% loss in weight was observed by *F. solani* MN201580.1 (SA17) when experimented with pre-treated films. Tribedi et al. (2013) observed that mineral oil accelerates microbial growth on polyethylene film, while Tween 80 reduces hydrophobic interactions, thereby lowering the attachment of microorganisms on the film. Although mineral oil might be modulating the hydrophobic interactions, in the present investigation, Tween 80 proved to be a suitable and effective chemical agent for enhancing the process of polymer bioremediation. The effect of nitric acid pre-treatment and the addition of Tween 80 significantly increases the degradation (Figure.40 and Table.25).

Tween 80: The best combination of pre-treatment and chemical substance for enhancing the degradation was found to be the 60°C heat treatment followed by 99.9% HNO3 and 0.05% Tween 80. Chemical surfactants like Tween 80 and Tween 20 assist the manganese peroxidase (MnP) enzyme in the degradation process (Ehara et al., 2000). Similar to the findings of previous experiments, the current investigation also observed a reduction in weight-loss percentage.

In just two weeks of incubation, a significant weight-loss of 38.8 ± 0.01% was achieved in the films. Subsequently, fungal accumulation appeared to take place on the film surface bringing about an increase in weight (Figure.40 and Table.25). Sangale et al. (2019) reported of such weight gain when

experimented with fungal strains *Aspergillus terreus* strain MANGF1/WL and *Aspergillus sydowii* strain PNPF15/TS isolated from the mangrove rhizosphere. When only nitric acid treatment was used without any addition of chemical substances, a weight-loss of 21.33% was observed when incubated with *F. solani* MN201580.1 (Table.11). This highlights the significant effect of the Tween 80 as an enhancer in polyethylene degradation, as it resulted in a 17.47% increase in the degradation rate. After four weeks of incubation, the weight-loss of the film was recorded as 37.8993 ± 0.002%, which reduced to 29.4523 ± 0.03% and 20.768 ± 0.01% in six and eight weeks, respectively. The percentage weight-loss was further recorded as 20.457 ± 0.004% and 20.777 ± 0.01% after testing for 12 and 16 weeks, respectively.

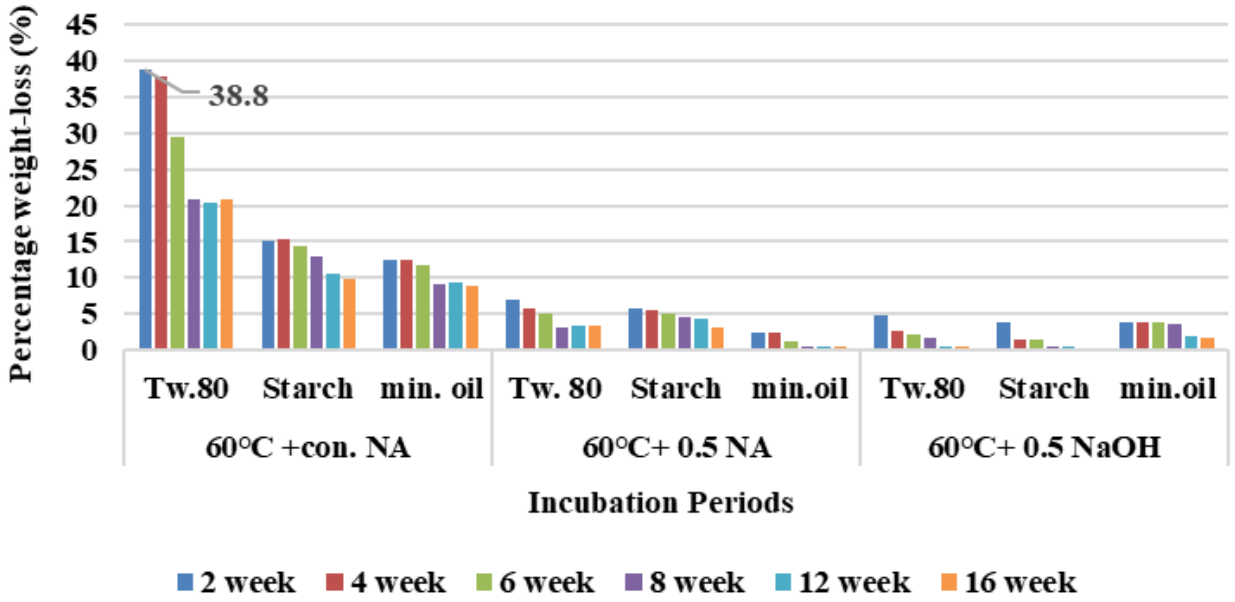

Figure.40. Percentage weight-loss of polyethylene films experimented by adding chemical enhancers (Tween80, Soluble Starch, Mineral oil)

The pre-treatment of PE films was found to play a vital role in the degradation process, as the degradation rates observed in the other two treatments were lower compared to the concentrated nitric acid treatment. The highest weight-loss of 6.89086 ± 0.0891% in the films was observed with the 60°C heat treatment followed by 0.5 N HNO_3 when incubated for two weeks. However, this weight-loss was almost 31% less when compared to the concentrated nitric acid treatment. Over time, the weight-loss percentage gradually decreased from 5.6663 ± 0.0356% in four weeks to 4.956 ± 0.0123% in six weeks. Following that the percentage weight loss remained almost consistent (ranging from 3.0092 ± 0.019% to 3.2456 ± 0.087% in the eighth, twelfth, and sixteenth week of incubation period).

The pre-treatment with the basic substance tween80 proved to be less efficient for polyethylene degradation. In two weeks of incubation, the maximum weight-loss of 4.66147 ± 0.024% was noted

in films treated with 60°C heat followed by 0.5 N NaOH. However, this percentage was notably lower compared to the other pre-treatment methods.

The films harvested in the 4th and 6th weeks showed weight reductions of 2.4997 ± 0.044% and 2.023 ± 0.05%, respectively. In the later stages of incubation, the weight-loss remained relatively low, ranging from 1.61442 ± 0.23% to 0.4243 ± 0.002%. Previous research reports have identified Tween 80 as an efficient enhancer for hydrocarbon breakdown (Albertsson et al., 1993; Cheng et al., 2017; Khan et al., 2017). *Psuedomonas* species with LDPE in the presence of 0.05% Tween 80 showed a weight-loss of 2% in 45 days (Tribedi et al., 2013). *F. solani* MN201580.1 in the present study, showed significant and more promising results.

Topographic changes such as black and brown patches, small holes on the surface, wrinkled PE samples, and mycelial growth were observed on the enhancer treated films. The colour of the harvested and SDS washed PE film changed from transparent to pale white and with intermittent black patches (Figure.41-arrow). These changes indicated some kind of deposition, which might be mycelial growth, explaining the increase in the final weight of the film. Upon removal after experimentation, the morphology of the film subjected to the experiment exhibited a curled and shrunken appearance in contrast to the controlled film. Similar observations were reported in PE film degraded by the marine fungus *Alternaria* species (Gao et al., 2021).

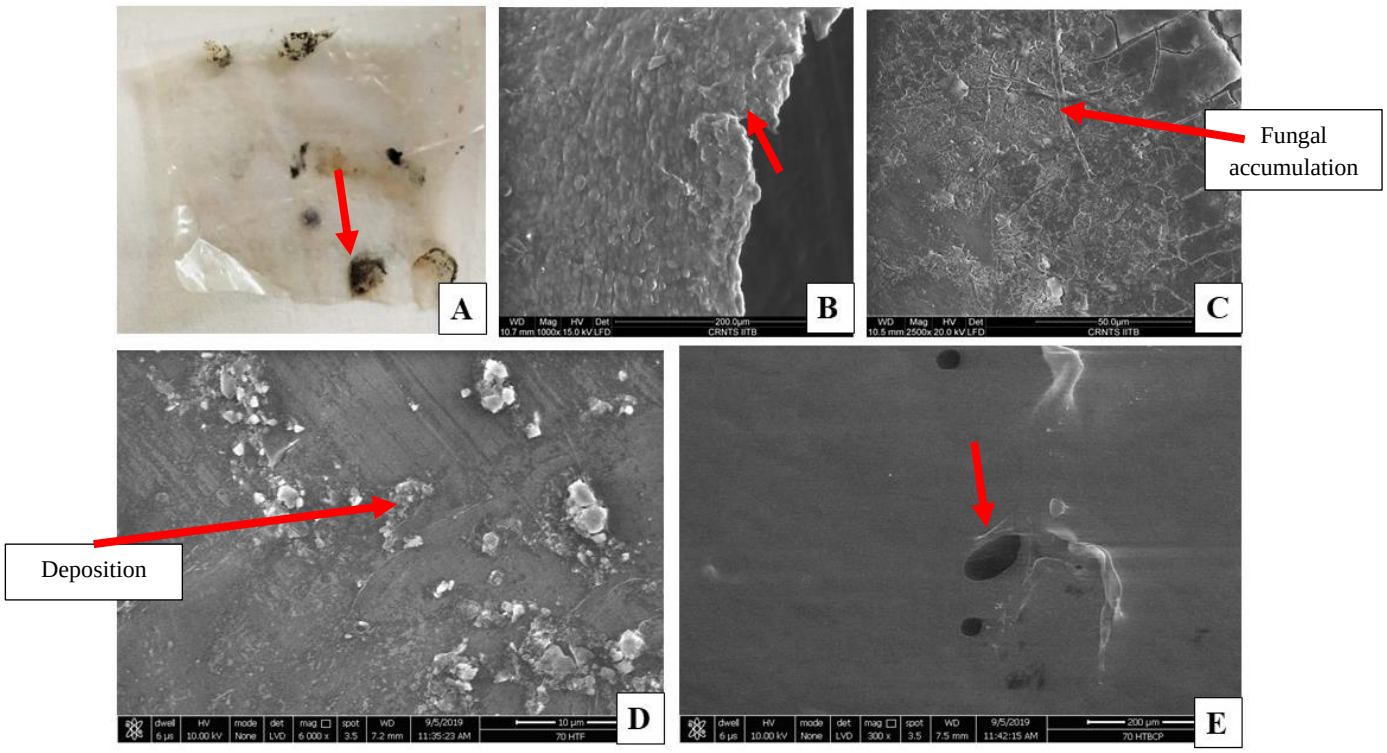

Figure.41. A- Experimented film after SDS wash displaying black spots
B, C, D & E-Scanning Electron Microscopic (SEM) images of polyethylene films: B- Crumpled and torn PE film (1000x); C & D- fungal and other deposition on PE film (2500x, 6000x); E- PE film with holes and folds (300x)

Scanning electron microscopy of the torn and crumpled film showed surface destruction (Figure.41-B-arrow). The damaged edge of the torn film and pits on the surface evidently indicates fungal degradation. Micrographs clearly depicts fungal accumulation and other depositions in the different regions of the film (Figure.41-C-arrow), which could be the reason behind the weight gain observed. ESEM analysis revealed holes and folds on the surface of the film (Figure.41-E-arrow). Compared to the previous experiment on screening conducted (Chapter-3), topography of the experimented film appeared to be altered due to the fungal association. This confirms the fact that the supplementation of Tween 80 surfactant has significantly enhanced the fungal degradation of polyethylene.

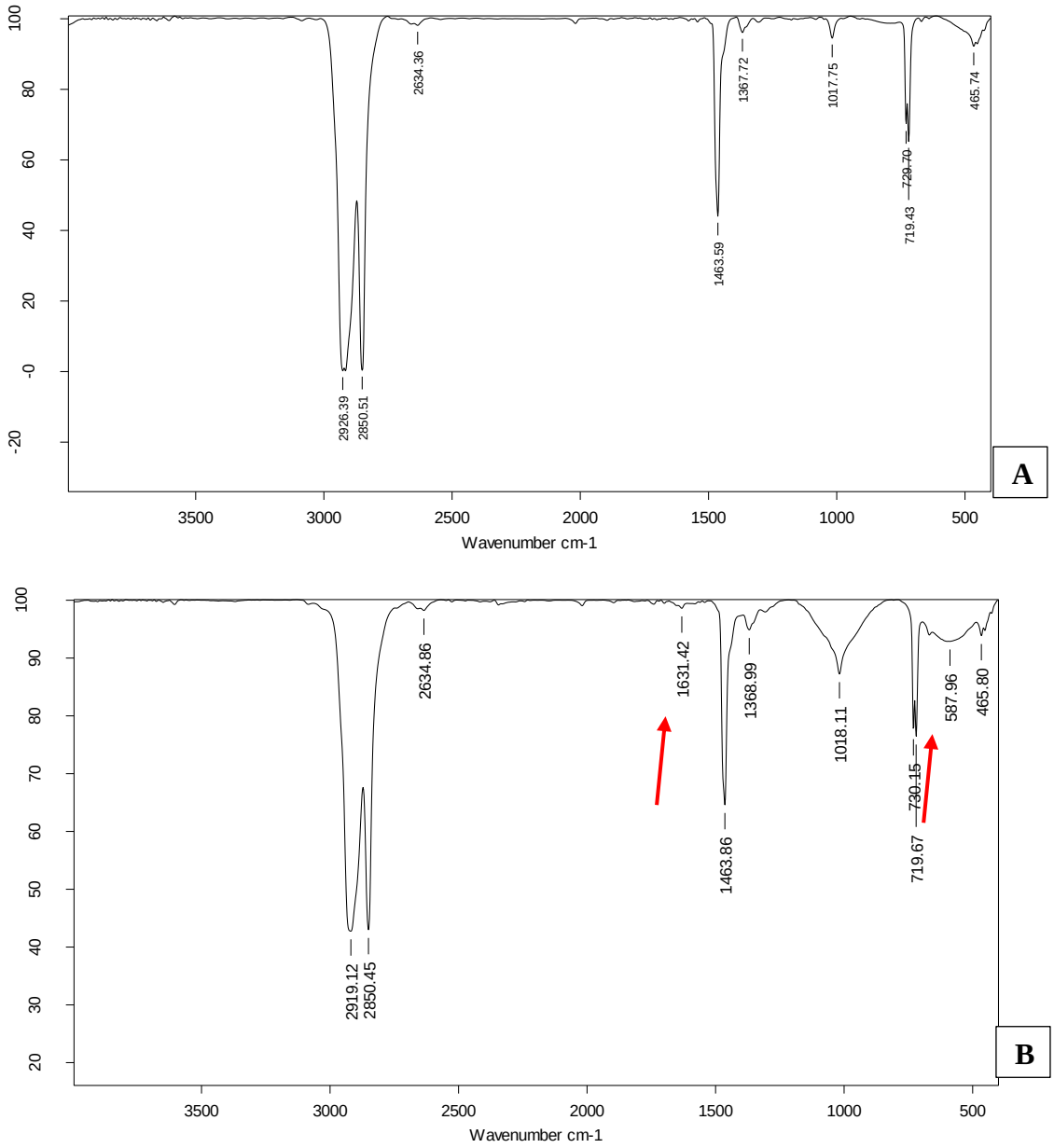

Figure.42. FTIR spectra of experimented PE films with Tween 80: A- Control PE film; B- Experimented PE film with maximum weight-loss

The FTIR analysis of the *F. solani* MN201580.1 (SA17) treated film revealed the formation of new peaks at 1631 cm^{-1} and 587 cm^{-1} (Figure.42-arrow). The appearance of these new peaks in the FTIR spectrum indicates the formation of new functional groups as a result of the degradation process. The degradation of polyethylene can lead to the breaking of carbon-carbon single bonds in the long chains, resulting in the formation of double bonds (C=C) and other new compounds. Typically, a peak at 587 cm^{-1} can be attributed to the presence of a C-H bending vibration, which may be related to the newly formed unsaturated compounds or other products of degradation. Similar phenomena were observed in the degradation of PS foam buried in sewage for 30 days by Umamaheswari & Murali (2013) and in the degraded LDPE strips with new peak developments in the region 721–1630 cm^{-1} reported by Skariyachan et al. (2016).

The carbonyl index, which is an indication of the presence of carbonyl groups, reduced from 1.04 (Control) to 0.98 (Experimented) due to the degradation process. This further confirms the occurrence of degradation in the experimented PE film with Tween 80, indicating the efficient degradation by *F. solani* MN201580.1 (SA17).

Table.25. Percentage weight-loss in experimented PE films with chemical enhancers

Pre-treatments		**60°C +con. NA**			**60°C+ 0.5 NA**			**60°C+ 0.5 NaOH**		
Enhancers		**Tween.80**	**Starch**	**Mineral oil**	**Tween.80**	**Starch**	**Mineral oil**	**Tween.80**	**Starch**	**Mineral oil**
Incubation Periods (weeks)	2	**38.8 ± 0.01**	15.2 ± 0.023	12.47 ± 0.012	6.89086 ± 0.0891	5.69646 ± 0.0654	2.46201 ± 0.011	4.66147 ± 0.024	3.763 ± 0.05	3.763 ± 0.09
	4	37.8993 ± 0.002	15.211 ± 0.011	12.462 ±0.09	5.6663 ± 0.0356	5.578 ± 0.083	2.4432 ± 0.039	2.4997 ± 0.044	1.439 ± 0.002	3.82 ± 0.003
	6	29.4523 ± 0.03	14.2999 ± 0.021	11.713 ± 0.0265	4.956 ± 0.0123	4.8965 ± 0.048	1.1713 ± 0.06	2.023 ± 0.05	1.363 ± 0.065	3.8296 ± 0.0034
	8	20.768 ± 0.01	13.001 ± 0.015	9.1734 ±0.034	3.0092 ± 0.019	4.4445 ± 0.09	0.568 ± 0.42	1.61442 ± 0.23	0.50 ± 0.001	3.602 ± 0.005
	12	20.457 ± 0.004	10.459 ± 0.01	9.3456 ± 0.092	3.2531 ± 0.076	4.2345 ± 0.056	0.534 ± 0.033	0.4243 ± 0.002	0.363 ± 0.0001	1.781 ± 0.002
	16	20.777 ± 0.01	9.82816 ± 0.02	8.898 ± 0.026	3.2456 ± 0.087	3.0001 ± 0.0754	0.523 ± 0.024	0.3419 ± 0.01	0.239 ± 0.002	1.60 ± 0.007

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

Soluble Strach:

The supplementation of 0.05% soluble starch reduced the degradation rate of the polyethylene films. Similar to the data obtained with Tween 80, heat and concentrated nitric acid treatments showed maximum degradation in replicates with starch supplementation. However, the weight of the films

gradually increased due to fungal growth on the surface, and eventually, films tested for longer time periods showed an increase in weight. The presence of starch in the medium probably served as a carbohydrate source for the increase in the fungal mycelial growth, thereby increasing the weight of PE film. The maximum weight-loss was recorded in two weeks, i.e., 15.2 ± 0.023%, by *F. solani* MN201580.1 (SA17), as shown in Figure.40 and Table.25. In the subsequent incubation periods, the percentage weight-loss remained almost similar, with 15.211 ± 0.011% and 14.2999 ± 0.021% weight-loss in 4 and 6 weeks, respectively. The percentage weight-loss in the experimented films then gradually reduced to 13.001 ± 0.015%, 10.459 ± 0.01%, and 9.82816 ± 0.02% in 8, 12, and 16 weeks of incubation, respectively.

In films with the pre-treatment of heat and 0.5 N nitric acid, results have been obtained similar to those with the Tween 80 inducer. The maximum weight-loss in this treatment was 5.69646 ± 0.0654% in two weeks and 5.578 ± 0.083% after 4 weeks. Subsequently, the weight-loss percentages were recorded as 4.8965 ± 0.048% in six weeks, 4.4445 ± 0.09% in eight weeks, and 4.2345 ± 0.056% in twelve weeks. The last harvested samples showed a weight-loss percentage of 3.0001 ± 0.0754% after sixteen weeks of incubation.

In the heat treatment followed by 0.5 N NaOH pre-treatment, the addition of 0.05% soluble starch in the growth medium resulted in negligible weight-loss. The maximum weight reduction in this experiment was recorded to be 3.763 ± 0.05% after two weeks, and the percentage reduced further to 1.439 ± 0.002% and 1.363 ± 0.065% in four and six weeks, respectively. As the incubation period increased, the weight-loss percentage continued to decrease, with values of 0.50 ± 0.001%, 0.363 ± 0.0001%, and 0.239 ± 0.002% in eight, twelve, and sixteen weeks of incubation, respectively.

Previous studies have reported significant enhancement of polymer degradation by inducing soluble starch (Geweely & Ouf, 2011; Shah et al., 2008). However, in contrast to those findings, the addition of starch in this experiment did not yield substantial results in terms of polyethylene degradation by *F. solani* MN201580.1 (SA17). The harvested films displayed topographic changes, as the color of the film turned slightly dull, with brownish to blackish patches were also observed. However, further SEM and FTIR analysis were not carried out for this experiment as significant results were not obtained with the addition of starch (weight-loss was comparatively negligible) and starch augmentation was excluded from further experimentation.

Mineral Oil:

In contrast to the earlier reported studies, in the present study the addition of mineral oil as an inducer did not yield the same level of effectiveness in enhancing the degradation process of polyethylene film. Hadar & Sivan (2004) reported a 50% improvement in polyethylene film degradation after 4 weeks with the addition of mineral oil to the medium, but in present study, the degradation achieved

by mineral oil was comparatively lower. While Tribedi and Sil (2012) reported a weight-loss of 14 ± 2% in untreated LDPE sheets inoculated with *Pseudomonas s*pecies in 45 days, which is higher than the weight-loss achieved supplemented mineral oil in present investigation.

The maximum weight-loss observed in this study was 12.47 ± 0.012% in two weeks when films were pre-treated with heat followed by concentrated HNO_3. This weight-loss percentage was substantially lower compared to the weight-loss achieved by the Tween 80 inducer (Figure.40 and Table.25). Over time, the weight-loss percentage further gradually decreased to 11.713 ± 0.0265% in six weeks, and 9.1734 ± 0.034% and 9.3456 ± 0.092% in the 8th and 12^{th} week of incubation, respectively.

The addition of mineral oil resulted in the lowest percentage weight reduction in all three types of pre-treatments. The films with heat and 0.5 N nitric acid pre-treatments showed the maximum weight-loss, with 2.46201 ± 0.011% and 2.4432 ± 0.039% after two and four weeks, respectively. Subsequently intervals, the harvested films exhibited negligible weight-loss, with only 1.1713 ± 0.06% in six weeks, and 0.568 ± 0.42% and 0.523 ± 0.024% in eight and sixteen weeks, respectively.

The pre-treatment with heat and sodium hydroxide on the film resulted in insignificant weight reduction with all three chemical inducers supplemented to the fungal inoculum. The highest weight-loss was achieved as 3.763 ± 0.09% and 3.82 ± 0.003% after 2 and 4 weeks, respectively, with a subsequent incubation period, the percentage weight-loss gradually reduced to 3.602 ± 0.005%, 1.781 ± 0.002%, and 1.60 ± 0.007% after eight, twelve, and sixteen weeks, respectively.

Crystal et al. (2023) reported positive effects of mineral oil supplementation in the culture media on biodegradation. Mineral oil is known to aid in the colonization on the film (Hadar et al., 2004). Similar to the other two inducers, mineral oil was able to change the topographic characteristics of the polyethylene film, significant weight reduction was not obtained. In the present set-up, Tween 80 proved to be more effective in promoting polyethylene degradation when supplemented to *F. solani* MN201580.1 (SA17). Hence for the further studies mineral oil supplementation as enhancer was excluded.

The abiotic augmentation experiment, after evaluating the effectiveness of three different inducers in speeding up the degradation process of polyethylene by *F. solani* MN201580.1. Tween 80 is considered as the most promising enhancer for promoting polyethylene degradation. This conclusion was further drawn by confirming the topographical features using E-SEM and FTIR analysis.

Key observations of the study

Fungal degradation of polyethylene was experimented with abiotic and biotic augmentation.

- Abiotic augmentation by supplementing with 0.05% tween 80 in medium was found to be significant for enhancing the degradation of polyethylene film (38.8 ± 0.01%) within two weeks.

- Biotic augmentation with fungal consortia did not show substantial degradation of polyethylene (4.1 ± 0.005%). Therefore, single culture technique was selected for further experimentation.