CHAPTER 4

Characterization and optimization of enzyme degradation by the selected fungi

Based on previous experiments, both *F. solani* MN201580.1 (SA17) and *A. oryzae* STR2 (SA15) strains were found to have the ability to degrade polyethylene through the release of extracellular enzymes. However, qualitative analysis of enzyme activity revealed that *F. solani* MN201580.1 (SA17) *was* more efficient in producing all five enzymes responsible for degradation. Therefore, *F. solani* MN201580.1 (SA17) was selected for further quantitative analysis, which showed that the optimum production of enzymes occurred between the 15^{th} and 25^{th} day of incubation.

The experiment was conducted in two parts. The first part aimed to determine the optimal conditions for maximum enzyme production by *F. solani* MN201580.1 (SA17) in the broth medium. This involved assessing the effects of incubation time, inoculum size, temperature, and pH. By optimizing these factors, it was aimed to improve the efficiency of enzyme production by the fungal strain.

In the second part of the experiment, the enzyme activity was evaluated in solid growth media, such as soil and mulch, to determine the degradation potential of *F. solani* MN201580.1 (SA17). This step was crucial in determining whether the fungal strain could effectively degrade polyethylene in real-world conditions outside the laboratory.

To assess the practical application of polyethylene degradation by *F. solani* MN201580.1 (SA17), PE films were tested in a natural medium consisting of soil and mulch. The enzyme activity was examined in this natural medium while keeping the optimized parameters identified in the earlier part of the experiment. In landfill areas, daily MSW is layered with soil, creating a mixture of soil and other degradable waste. This mixture could serve as a medium for fungal growth. Mulch, on the other hand, contains compostable components of MSW, such as kitchen waste, rotten fruits and vegetables, peels of fruits and vegetables, paper waste, dry leaves, and branches.

Therefore, a combination of soil and mulch was chosen as the solid growth medium for the experiment. By testing PE films in this natural medium, it could assess the potential of *F. solani* MN201580.1 (SA17) to degrade polyethylene under realistic conditions that mimic those found in landfill areas.

METHODOLOGY

To gain insight into the degradation process by *F. solani* MN201580.1 (SA17) species, characterization and optimization of fungal enzymes were performed to understand the effect of inoculum size of fungus, incubation time & temperature and pH level of the culture media at which maximum enzyme activity is detected. The experiment was conducted in three different culture media to measure the respective enzyme activities. The ligninolytic activity was measured in Malt extract

medium, proteolytic enzyme in Casein broth, and lipolytic in MS medium. Additionally, the fungal biomass was studied for each parameter to understand the impact on mycelial growth. All investigations were performed with three replicates for each incubation period and results reported as the mean of replicates to ensure statistical rigor.

Incubation Time

The culture replicates were kept for nine incubation period starting from 3rd day to 35th day at 30^{o} C with 5day interval followed by enzyme extraction to determine the optimum incubation period. Optimum incubation time was determined under all physical parameters to understand ideal period for the enzyme secretion under different factors.

Inoculation Size

In order to check the effect of fungal inoculum size on enzyme production, the experiment was executed with single 9mm disc and three 9mm discs of 10 days old culture along with control. All cultures were kept for nine incubation periods while maintaining all other parameters at their optimum level. The optimum size of an inoculum was fixed for following experiments.

Temperature

To examine the production of these enzymes at different temperatures, culture replicates were incubated at temperatures ranging from 25ºC to 40ºC for a desired period of time. Enzyme activity was assayed by extracting enzymes at each incubation period. To assess enzyme stability, extracts were incubated at 10ºC to 60ºC temperature range for 60 minutes. The optimum temperature achieved through this process was fixed for subsequent experiments.

pH level

Fungal growth and enzyme production capabilities were examined in culture media prepared with pH values (4 to 14) using 0.1 M NaOH or 0.1 N HCl and enzyme assays were performed. To determine the optimal pH for enzyme stability, crude enzyme extracts were incubated in respective buffers for each pH level for 60 minutes.

Optimization of enzyme extraction methodology for solid state medium

All five enzymes were determined in medium containing soil and mulch by inoculating *F. solani* MN201580.1 (SA17) and optimal parameters were maintained for the growth.

A. **Preparation of Fungal enzymes:** To prepare the culture replicates, 50 gm of garden soil and 50 gm of mulch sterilized at 121º C for 30 minute and aseptically moistened with 50ml of sterile distilled water to create 60% moisture level. The culture bottles were then inoculated with 9mm disc of 10-day-old culture grown on Potato Dextrose agar (PDA) medium and incubated at 30º C for each desired incubation period. At each incubation period, cultures were harvested and enzymes were extracted for quantification.

B. Enzyme extraction: To standardize the suitable methodology for extracting all fungal enzymes, two different extraction methods were performed**.** Culture replicates were harvested at the desired incubation period and enzymes were extracted in solvents, following a modified protocol. For extracting enzymes in solid state medium, 50ml of solvent was added to each bottle and the mixture was shaken on a rotary shaker (150 rpm) for 60 minutes at room temperature. The mixture was then squeezed through a double layer muslin cloth and centrifuges at 5000 rpm for 20 minutes. The clear supernatant was collected and used as crude enzyme extract for further enzyme assays (Falony et al., 2006; Mahadik et al., 2022; Christakopoulos et al., 1998).

Two types of solvents were added to determine the suitable solution for enzyme extractions:

(i) Distilled water (Christakopoulos et al., 1998; Falony et al., 2006; Niladevi et al., 2007).

(ii) Buffers: Three buffer solutions were prepared for respective enzymes. For the extraction of laccase & MnP enzymes 50mM sodium-acetate buffer (pH 5.0), 50 mM Tris-HCl used to extract lipase & esterase enzymes and protease enzyme was extracted by 10% (V/V) Ethanol water mixture (Tunga et al., 1999; Perdani et al., 2020).

RESULT & DISCUSSION

The production as well as stability of each enzyme strongly depends on incubation time & temperature, pH of the growth media and inoculum size. Therefore, determining the optimal parameters for each enzyme is crucial in enhancing their enzymatic degradation capabilities of polyethylene (PE) film.

Determination of optimal inoculum size

Inoculum size is an important parameter among all parameters affecting the production of fungal enzymes, as it affects proliferation of cells to consume the substrate and to produce enzyme. Higher inoculum size may enhance the biomass production but it may adversely affect enzyme secretion. As pointed out by Ramachandran et al. (2004), maintaining a balance between the increasing biomass and availability of required nutrients would yield an ideal enzyme secretion, indicating which that a smaller inoculum size may be more beneficial for enzyme production in this particular experimental setup. To check the effect of fungal inoculum size on the enzyme production, the experiment was executed with single disc and three discs of inoculum in three culture media, kept for nine incubation period starting from 3days to 35 days. In all five enzymes, single disc replicates produced more amount of enzymes than three discs inoculated culture replicates.

Laccase activity by single disc was observed to be increasing rapidly after 10^{th} day of incubation, with the activity at its peak on 25^{th} day and then further decreasing up to 35^{th} day. Laccase production showed a constant increase up to the 25th day (3.41±0.7 U/ml) and then declined to 2.34±0.2 U/ml on the 35^{th} day, as shown in Figure.29-A. When the culture replicates were inoculated with 3-disc, the laccase activity increased up to 1.19±0.50 U/ml on the 5^{th} day and then slightly reduced to 0.81±0.22

U/ml on the 10th day. The activity gradually increased again with peak activity on the 20th day (1.58±0.3 U/ml). Laccase production seemed to be reduced after its peak, which showed a gradual increase up to 1.9±0.12 U/ml on the 35th day of incubation. When the inoculum sizes were compared, the single disc inoculum showed a smooth increase and decrease of enzyme activity, while the three-disc inoculum showed an increase followed by reduced activity up to 15th day and then a significant increase by 20th day (Figure.29-A).

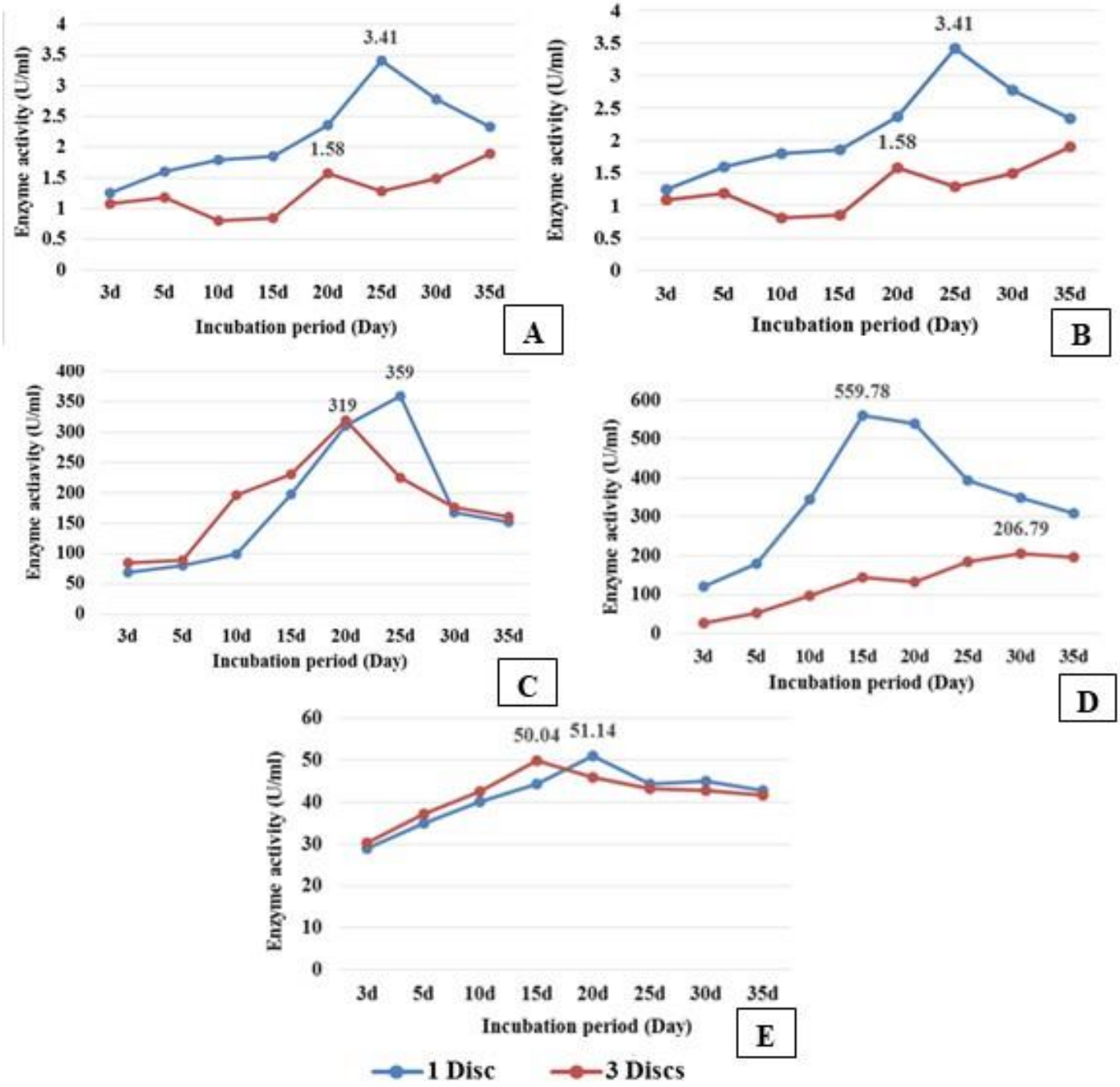

Figure.29. Quantitative analysis of enzyme activities by two inoculum sizes of *F. solani*
A- Laccase activity; **B-** MnP activity; **C-** Protease activity; **D-** Esterase activity; **E-** Lipase activity

Although the cultures inoculated with a single disc produced enzymes slowly, they released a greater amount of enzymes than cultures inoculated with three discs. The highest production of laccase was observed on the 25th day in the cultures with a single disc, while replicates with three inoculum discs showed high production on the 20th day. There was a significant difference in enzyme production influenced by lower inoculum size. Laccase secretion decreased in the three-disc inoculum cultures due to rapid depletion of available nutrients, resulting in a decline in activity.

The replicates inoculated with one disc showed 3.41±0.7 U/ml of laccase enzyme, while cultures with three discs showed 1.58±0.3 U/ml enzyme. Patel et al. (2009) found that the optimal inoculum size for highest laccase production (2700 U/g) by *Pleurotus ostreatus* strain was five discs (8mm). Other studies have also reported higher amounts of biomass and laccase activity in media inoculated with smaller amounts of inoculum for *A. parasiticus* and *P. ostreatus* (Etcheverry et al., 1996; Hu & Duvnjak, 2004).

Table.15. Enzyme production by two inoculum sizes of *F. solani* MN201580.1 at various incubation periods

Incubation period		3days	5days	10days	15days	20days	25days	30days	35days
Laccase (U/ml)	1 Disc	1.25±0.14	1.6±0.08	1.8±0.11	1.86±0.15	2.37±0.08	**3.41±0.7**	2.78±0.05	2.34±0.2
	3 Disc	1.08±0.20	1.19±0.50	0.81±0.22	0.85±0.18	**1.58±0.3**	1.29±0.5	1.49±0.41	1.9±0.12
MnP (U/ml)	1 Disc	0.12±0.02	0.24±0.05	0.14±0.03	**0.2±0.1**	0.11±0.02	0.15±0.09	0.18±0.09	0.18±0.06
	3 Disc	0.1±0.06	0.11±0.05	0.14±0.08	0.1±0.05	**0.19±0.04**	0.18±0.1	0.14±0.03	0.16±0.03
Protease (U/ml)	1 Disc	69±5.74	80±3.65	98.5±1.91	197.5±3.41	310.5±3.41	**359±2.58**	167.5±3	151.5±3.41
	3 Disc	84.5±4.12	89±2.58	196±4.32	231±6.21	**319±2.58**	225±4.16	176.5±3.42	162.5±5.97
Esterase (U/ml)	1 Disc	120.5±1.25	178.93±1.50	343.86±2.08	559.78±1.62	538.9±1.48	393.95±1.13	348.73±1.13	308.45±1.01
	3 Disc	27.74±1.19	54.29±1.44	98.37±0.77	145.23±1.95	133.72±2.7	185.34±1.23	206.79±1.39	195.95±1.37
Lipase (U/ml)	1 Disc	28.95±1.44	35.09±2.06	40.12±2.17	44.32±1.09	**51.14±1.76**	44.47±1.23	44.93±1.31	42.73±0.68
	3 Disc	30.28±2.18	37.27±0.43	42.5±3.75	**50.04±2.88**	45.87±0.68	43.18±1.71	42.73±0.81	41.63±1.68

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

MnP enzyme activity produced by both inoculum size showed slightly different patterns. The results show that both single-disc and three-disc inoculum equally showed MnP enzyme activity, but with slightly different patterns. The single-disc inoculum produced higher enzyme activity on 5th day (0.24±0.05 U/ml) and further increase on 15th day (0.2±0.1 U/ml), while the three-disc inoculum produced almost equal amounts of enzyme on 3rd day (0.11±0.05 U/ml) and 10th day (0.14±0.08 U/ml) compared to the single-disc inoculum. However, the enzyme activity peaked on 20th day (0.19±0.04 U/ml) in culture replicates with three-disc inoculum, which is similar to the activity by

single-disc inoculum. Both inoculum sizes showed a slight increase in enzyme activity in the next incubation periods, but the production reduced after 20th day and reached 0.16±0.03 U/ml on 35th day of incubation.

The data showed that although the amount of MnP enzyme produced was almost similar in both inoculum sizes, cultures inoculated with three discs of *F. solani* MN201580.1 (SA17) took 20 days to show 0.19±0.04 U/ml MnP enzyme activity, whereas a single disc inoculum produced 0.24±0.05 U/ml amount of MnP in five days (Figure.29-B & Table.15).

The **protease enzyme** activity increased rapidly in both the cultures with 1-disc and 3-disc inoculum. In the 1-disc inoculum, the activity increased from 69±5.74 U/ml on the 3rd day to 359±2.58 U/ml on the 25th day. In the 3-disc inoculum, the activity was recorded as 84.5±4.12 U/ml on the 3rd day and gradually increased to 319±2.58 U/ml on the 20th day as showed in Table.15 & Figure.29-C. The activity dropped to 151.5±3.41 U/ml and 162.5±5.97 U/ml on the 35th day in the 1-disc and 3-disc inoculum respectively. The activity was almost similar in both inoculum sizes on the initial 3rd and 5th day and later on the 30th and 35th day. Overall, cultures with the 3-disc inoculum had a slower increase in protease activity, but it ultimately reached a similar peak of activity as the 1-disc inoculum.

Table.16. Fungal biomass of *F. solani* MN201580.1 produced in culture broths during different incubation period

Incubation period		3days	5days	10days	15days	20days	25days	30days	35days
Ligninolytic (gm)	1 Disc	0.15±0.02	0.155±0.005	0.19±0.03	0.23±0.04	**0.815±0.01**	0.415±0.02	0.415±0.02	0.65±0.05
	3 Disc	0.25±0.03	0.23±0.03	0.23±0.04	0.99±0.09	**1.05±0.06**	0.62±0.05	0.57±0.04	0.61±0.05
Proteolytic (gm)	1 Disc	0.12±0.01	0.24±0.005	0.44±0.05	**0.47±0.03**	0.4±0.005	0.41±0.01	0.48±0.01	0.33±0.01
	3 Disc	0.19±0.01	0.33±0.005	0.5±0.02	**0.55±0.02**	0.47±0.02	0.35±0.005	0.34±0.01	0.32±0.005
Esterolytic & Lipolytic (gm)	1 Disc	0.76±0.06	0.83±0.04	0.755±0.15	0.85±0.04	**0.93±0.18**	0.85±0.07	0.8±0.15	0.68±0.08
	3 Disc	0.94±0.08	0.90±0.07	0.82±0.06	0.9±0.09	**0.99±0.02**	0.85±0.07	0.89±0.12	0.77±0.02

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

Uyar and Baysal (2004) reported that increasing inoculum size could lead to a decline in protease enzyme secretion due to nutrient depletion in the medium. In the present study, a similar trend was observed, with enzyme production decreasing with higher inoculum size but remaining almost equivalent to the small inoculum size. Protease enzyme activity was consistently higher in the 3-disc inoculum size than in the single-disc replicates on each incubation period (Figure.29-C, Table.15). However, the enzyme production peaked on the 20th day with 319±2.58 U/ml in the 3-disc replicates, while in the single-disc replicates, the production slowly increased and peaked on the 25th day with 359±2.58 U/ml.

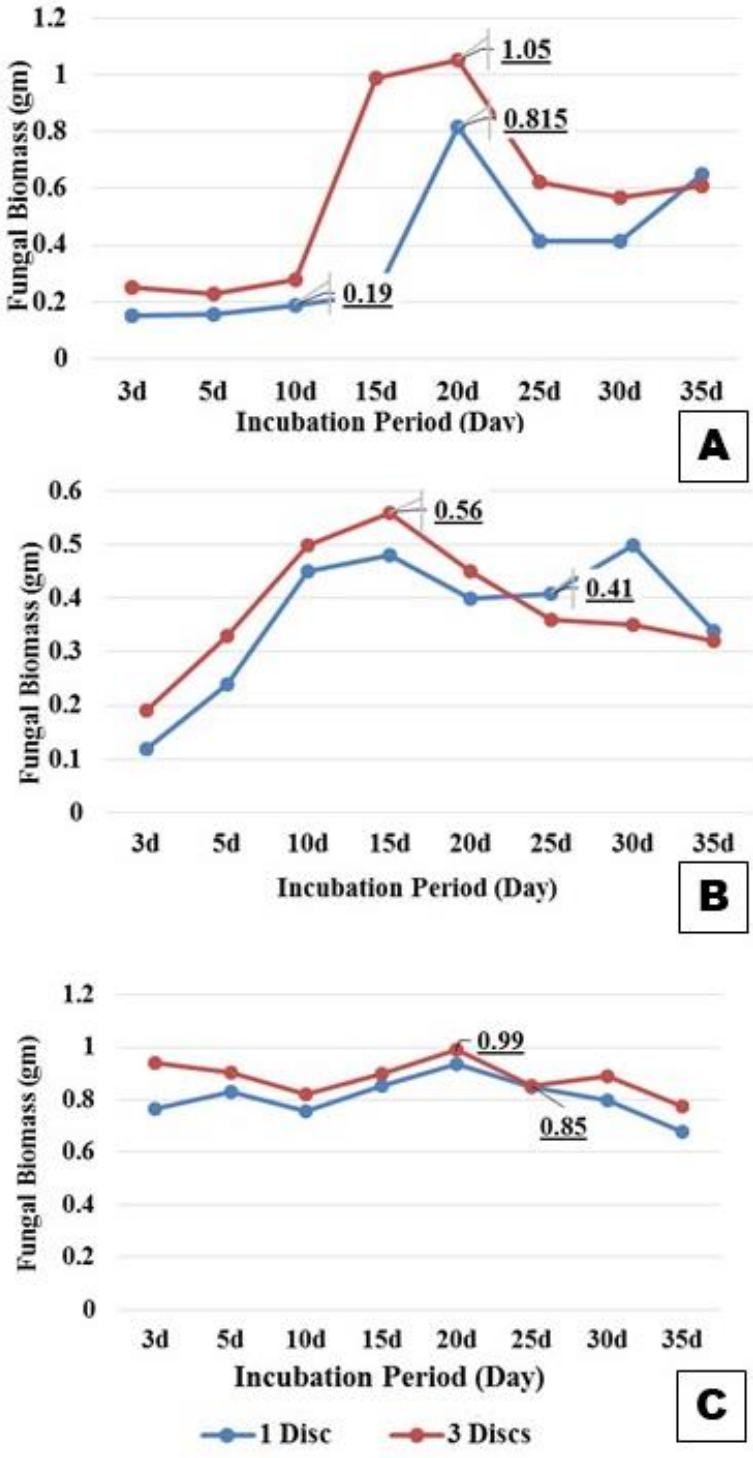

Figure.30. Fungal biomass obtained in 3 enzyme substrate media with 2 different inoculum size: A- Malt extract medium**; B-** Casein broth medium**; C-** MS Medium

The **esterase enzyme** activity was higher in the 1-disc inoculum size as compared to the 3-disc inoculum size. In the 1-disc inoculum size, the activity increased rapidly from 178.93±1.50 U/ml on 5th day to 559.78±1.62 U/ml on 15th day (Figure.29-D and Table.15). On the other hand, in the culture replicates with 3-disc, the activity started with a low value of 27.74±1.19 U/ml on 3rd day and

gradually increased to 145.23±1.95 U/ml on 15th day and further increased to 206.79±1.39 U/ml on 30th day of incubation.

Just like laccase activity, esterase production seemed to be influenced by the size of an inoculum. The enzyme activity was peaked after 15th day of inoculation in both the inoculum sizes, single inoculum disc was able to produce 559.78±1.62 U/ml of enzyme and inoculum size of 3 discs showed less amount of activity (145.23±1.95 U/ml). Similar to laccase activity, the production of esterase enzyme also appeared to be influenced by the inoculum size. The enzyme activity in both inoculum sizes reached its peak after 15 days of inoculation. The single inoculum disc produced a higher amount of enzyme activity at 559.78±1.62 U/ml, while the inoculum size of 3 discs showed a lower amount of activity at 145.23±1.95 U/ml.

The **lipase enzyme** activity in both single disc and 3-disc inoculum increased from the initial days of incubation. In the single disc inoculum, the lipase activity increased from 28.95±1.44 U/ml on the 3rd day to 51.14±1.76 U/ml on the 20th day, before reducing to 42.73±0.68 U/ml on the 35th day (Figure.29-E and Table.15). In contrast, the lipase activity in the 3-disc inoculum was slightly higher than that of the single disc inoculum until the 10th day, with 30.28±2.18 U/ml produced on the 3rd day and increased to 50.04±2.88 U/ml on the 15th day, before declining to 41.63±1.68 U/ml on the 35th day. The production of lipase enzyme was considerable between 10 days to 25 days of incubation in both inoculum sizes.

Similar to other enzymatic activities, no significant difference was observed between two inoculum sizes for lipolytic enzyme activity. Extracellular lipase was produced in almost equal amount in both the inoculum sizes. Single disc of *F. solani* MN201580.1 (SA17) released 51.14±1.76 U/ml amount of lipase on 20th day after inoculation, and 3 discs showed 50.04±2.88 U/ml enzyme activity on 15th day of incubation. Similarly, to MnP and Protease enzyme activities, there was no significant difference observed in the lipolytic enzyme activity between the two inoculum sizes. The extracellular lipase enzyme was produced in almost equal amounts in both the inoculum sizes.

Experiment was conducted to determine the relationship of fungal biomass obtained with two different inoculum size when grown on the different enzyme substrate medium. In the 1-disc replicates with MEB medium, the fungal biomass increased from 0.15±0.02 gm on the 3rd day to 0.815±0.01 gm on the 20th day, after which it decreased. On the other hand, in the 3-disc replicates, the fungal biomass increased from 0.25±0.03 gm on the 3rd day to 1.05±0.06 gm on the 20th day, after which it also decreased, but not as drastically as in the 1-disc replicates. By the 35th day of incubation, the fungal biomass in the 3-disc replicates was 0.61±0.05 gm, which was still higher than the initial biomass on the 3rd day. Furthermore, mycelial growth of *F. solani* MN201580.1 (SA17) was more pronounced in replicates with three discs inoculum than in single disc replicates, with the fungal biomass reaching

0.815±0.01 gm for the single disc inoculum and 1.05±0.06 gm for the three-disc inoculum on the 20th day in MEB medium (Figure.30-A & Table.16).

Table.16 depicts fungal biomass obtained in casein culture medium and indicates that the mycelial growth was higher in the 3-disc inoculum size (0.55±0.02 gm) than in the single-disc inoculum size (0.47±0.03 gm), and both inoculum sizes reached maximum growth on the 15th day of incubation. Fungal biomass recorded as 0.12±0.01 gm in replicates with 1-disc inoculum, in 3-disc replicates 0.19±0.01 gm biomass was produced on 3rd day. The fungal growth reduced to 0.33±0.01 gm and 0.32±0.005 gm on 35th day in 1-disc and 3-disc inoculum size respectively.

It seems that in MS broth culture medium, there was not much difference in mycelial growth between the two inoculum sizes. On the 3rd day of incubation, 0.76±0.06 gm and 0.94±0.08 gm of fungal growth was produced by 1-disc and 3-disc inoculum, respectively. The fungal growth gradually increased until the 20th day, with maximum growth recorded as 0.93±0.18 gm and 0.99±0.02 gm in 1-disc and 3-disc culture replicates, respectively. However, the growth slowly decreased to 0.68±0.08 gm and 0.77±0.02 gm by single disc and three-disc inoculum, respectively, as shown in Table.16 and Figure 30-C.

In conclusion, the production of laccase and esterase enzymes appeared to be highly influenced by the inoculum size. The amount of enzyme activity was found to be significantly different between the two inoculum sizes for these two enzymes. However, there was no significant difference observed in lipolytic enzyme activity between the two inoculum sizes. Overall, the results suggest that inoculum size can affect the growth and enzyme production of *F. solani* MN201580.1 (SA17), with larger inocula leading to delayed enzyme production but increased fungal biomass.

Determination of optimum temperature for maximum enzyme activity

The selected range of incubation temperature was decided on the basis of seasonal temperatures of the country and previous investigation reports. Experiments were conducted for 25°C, 30°C, 35°C and 40°C temperature. Figure.31 depicts the variations observed in the different enzyme activities when subjected to four different temperatures. In this study, significant enzyme activity was observed at 30ºC temperature, and both fungal biomass and enzyme activity were found to decrease with higher temperatures.

The **laccase enzyme** production was highest at 35°C temperature, while it was low at 25°C, 30°C, and 40°C. The activity remained stagnant until the 15th day of incubation, after which it showed a quick increase in the graph. On the 3rd day of incubation, the fungus produced 1.28±0.50 U/ml and 1.25±0.14 U/ml of enzyme activity at 25°C and 30°C, respectively. In comparison, it produced only 0.59±0.06 U/ml and 0.46±0.03 U/ml of enzyme activity at 35°C and 40°C, respectively. However, in later days, at 35°C temperature, the enzyme activity increased significantly from 3.93±0.02 U/ml on the 15th day

to 14.57±0.04 U/ml after 25 days of inoculation. The enzyme activity was slow and less at 25°C, 30°C, and 40°C, as enzyme production showed a slow increase from 1.27±0.46 U/ml, 1.6±0.08 U/ml, and 0.49±0.03 U/ml (5th day) to 2.11±0.13 U/ml, 3.41±0.78 U/ml, and 0.67±0.03 U/ml on the 25th day, respectively. Laccase production was observed to decrease after reaching maximum activity on the 25th day, with 1.75±0.22 U/ml, 2.34±0.2 U/ml, 11.22±0.03 U/ml, and 0.51±0.02 U/ml of enzyme production recorded on the 35th day at 25°C, 30°C, 35°C, and 40°C temperatures, respectively.

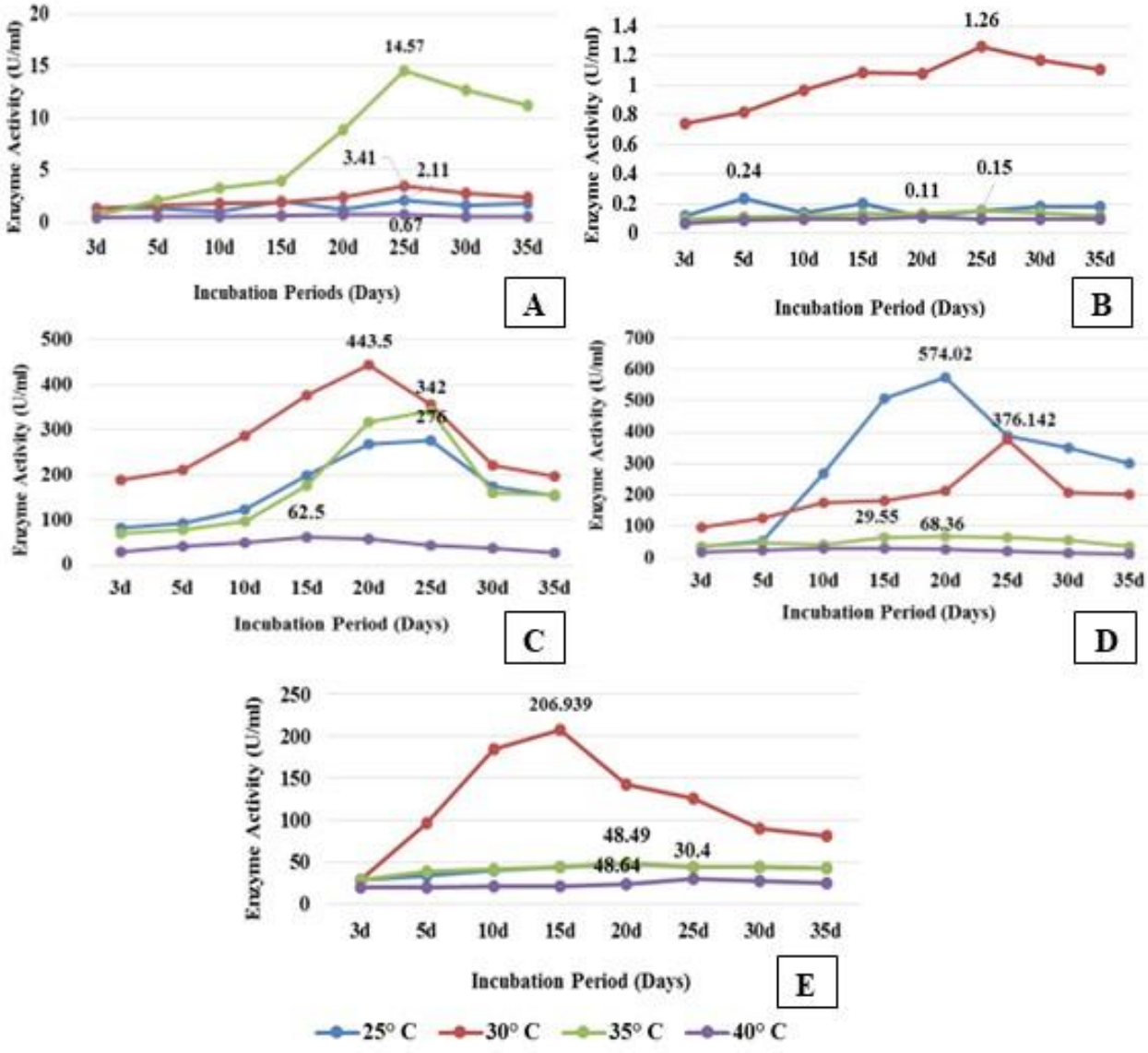

Figure.31. Quantitative analysis of enzyme activities by *F. solani* at different temperatures

A- Laccase activity; **B-** MnP activity; **C-** Protease activity; **D-** Esterase activity; **E-** Lipase activity

The optimal temperature for fungal laccase activity can vary significantly from one strain to another. While the temperature range of 50°C-70°C is most suitable for laccase activity in most fungal species (Baldrian 2005; Luisa et al., 1996), a study on laccase activity from *Fusarium* sp. found that the highest enzyme activity, at 0.311 U/ml, was observed at 25°C, with the enzyme becoming

progressively less active at higher temperatures (Sehga et al., 2020). In comparison to that, *F. solani* MN201580.1 (SA17) showed promising results, with the ability to secrete 14.57 ± 0.04 U/ml of laccase enzyme at 35°C. However, the amount of laccase activity decreased with decreasing temperature, with 2.11 ± 0.13 U/ml of the enzyme produced at 25°C as shown in Table.17 & Figure.31-A. The optimum temperature range for the crude laccase enzyme was found to be 35°C, with the enzyme retaining its activity within a temperature range of 25°C to 50°C.

Table.17. Enzyme production by *F. solani* MN201580.1 at four different temperatures

Enzyme Activity	Incubation period	Temperature			
		25° C	**30° C**	**35° C**	**40° C**
Laccase (U/ml)	**3d**	1.28 ± 0.50	1.25 ± 0.14	0.59 ± 0.06	0.46 ± 0.03
	5d	1.27 ± 0.46	1.6 ± 0.08	2.06 ± 0.34	0.49 ± 0.03
	10d	0.99 ± 0.35	1.8 ± 0.11	3.25 ± 0.06	0.55 ± 0.02
	15d	1.95 ± 1.42	1.86 ± 0.15	3.93 ± 0.02	0.63 ± 0.04
	20d	1.22 ± 0.04	2.37 ± 0.08	8.89 ± 0.01	0.67 ± 0.03
	25d	2.11 ± 0.13	3.41 ± 0.78	**14.57 ± 0.04**	0.67 ± 0.03
	30d	1.63 ± 0.33	2.78 ± 0.05	12.63 ± 0.04	0.56 ± 0.02
	35d	1.75 ± 0.22	2.34 ± 0.2	11.22 ± 0.03	0.51 ± 0.02
MnP (U/ml)	**3d**	0.12 ± 0.02	0.74 ± 0.01	0.09 ± 0.009	0.06 ± 0.004
	5d	0.24 ± 0.16	0.82 ± 0.01	0.11 ± 0.003	0.08 ± 0.005
	10d	0.14 ± 0.03	0.97 ± 0.02	0.12 ± 0.009	0.09 ± 0.005
	15d	0.11 ± 0.02	1.09 ± 0.02	0.13 ± 0.006	0.10 ± 0.005
	20d	0.11 ± 0.02	1.08 ± 0.04	0.13 ± 0.006	0.11 ± 0.005
	25d	0.10 ± 0.027	**1.26 ± 0.04**	0.15 ± 0.004	0.10 ± 0.006
	30d	0.09 ± 0.01	1.17 ± 0.03	0.14 ± 0.008	0.10 ± 0.003
	35d	0.18 ± 0.06	1.11 ± 0.02	0.12 ± 0.003	0.09 ± 0.003
Protease (U/ml)	**3d**	82.5 ± 1.91	188.5 ± 1	69 ± 1.15	28.5 ± 1
	5d	92 ± 1.63	210.5 ± 1.91	77 ± 1.15	40.5 ± 1
	10d	124 ± 1.63	286 ± 1.63	96 ± 1.63	49 ± 2
	15d	198 ± 1.63	376 ± 1.63	176 ± 1.63	62.5 ± 1.91
	20d	268 ± 2.82	443.5 ± 1.91	317 ± 1.15	57.5 ± 1.91
	25d	276.5 ± 1.91	**356 ± 1.63**	342 ± 1.63	43.5 ± 1
	30d	174.5 ± 1.91	222 ± 1.63	160 ± 1.63	37 ± 1.15
	35d	154 ± 1.63	197 ± 1.15	155.1 ± 1.16	26 ± 1.63
Esterase (U/ml)	**3d**	35.85 ± 2.77	98.43 ± 0.39	37.44 ± 4.3	17.41 ± 0.33
	5d	54.01 ± 0.17	124.41 ± 1.07	50.71 ± 0.48	25.13 ± 0.89
	10d	268.85 ± 0.25	170.08 ± 1.73	41.75 ± 0.512	28.08 ± 0.55
	15d	509.52 ± 0.62	155.22 ± 0.86	64.22 ± 0.27	29.55 ± 0.5
	20d	**574.02 ± 0.51**	213.88 ± 1.15	68.36 ± 0.33	25.7 ± 1.07
	25d	390.15 ± 0.5	375.63 ± 0.63	66.15 ± 0.27	20.87 ± 0.27
	30d	350.15 ± 2.42	205.99 ± 0.39	56.16 ± 0.37	15.37 ± 0.5
	35d	301.19 ± 0.84	204.58 ± 0.48	34.49 ± 0.27	13.21 ± 0.25
Lipase (U/ml)	**3d**	28.81 ± 0.48	29.28 ± 0.61	28.85 ± 1.53	19.47 ± 0.21
	5d	34.04 ± 0.19	95.85 ± 0.21	39.28 ± 0.59	19.71 ± 0.13
	10d	40.08 ± 0.17	184.81 ± 0.96	40.90 ± 1.96	21.03 ± 0.17
	15d	44.31 ± 1.09	**206.94 ± 0.39**	43.71 ± 0.72	21.63 ± 0.14
	20d	48.49 ± 1.10	142.77± 0.40	48.64 ± 1.32	24.02 ± 0.19
	25d	44.7 ± 1.23	125.16 ±0.37	44.70 ± 1.23	30.42 ± 0.18
	30d	43.94 ± 1.33	90.5 ±0.24	43.98 ± 1.32	27.35 ± 0.22
	35d	42.73 ± 0.68	80.76 ±0.17	42.73 ± 0.68	25.31 ± 0.17

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

MnP enzyme activity by *F. solani* MN201580.1 (SA17) is highly dependent on the temperature range. The maximum activity was observed at 30°C, with 0.74±0.01 U/ml of enzyme activity on the third day. In contrast, lower levels of enzyme production were observed at other temperatures, with only 0.12±0.02 U/ml, 0.09±0.009 U/ml, and 0.06±0.004 U/ml produced at 25°C, 35°C, and 40°C, respectively. At 30°C, the enzyme activity gradually increased from 0.82±0.01 U/ml on the fifth day to a maximum of 1.26±0.04 U/ml on the 25th day, followed by a slight reduction to 1.17±0.03 U/ml and 1.11±0.02 U/ml on the 30th and 35th day, respectively. At 25°C, a maximum activity of only 0.24±0.16 U/ml was observed on the fifth day, which decreased to 0.09±0.01 U/ml on the 30th day, but then showed a two-fold increase on the 35th day after a reduced activity on the 30th day. At 35°C and 40°C, enzyme activity of 0.11±0.003 U/ml and 0.08±0.005 U/ml was recorded on the fifth day, which gradually increased to 0.15±0.004 U/ml (25th day) and 0.11±0.005 U/ml (20th day), respectively.

Previous study has shown that the incubation temperature can have a significant impact on the activity of ligninolytic enzymes, as well as individual enzymes produced by a given strain of fungi (Vyas et al. 1994). For instance, *Pleurotus* species have been found to exhibit maximum laccase activity (1.2-1.5 U/ml) when grown at temperatures between 25°C and 30°C, while the highest production of MnP enzyme (1.5-2 U/ml) was observed at temperatures ranging from 20°C to 30°C (Zadrazil et al., 1999). The study also mentioned laccase enzyme activity peaked during the first week of incubation, while MnP enzyme production reached its highest level during the second week. Both enzymes showed a gradual decline in activity by the fourth week of incubation. In the present study, laccase and MnP enzyme production from *F. solani* MN201580.1 (SA17) peaked during the third week of incubation and gradually decreased thereafter, as indicated in Figure.31.

Zadrazil et al. (1999) reported MnP activity by *Dichomitus squalens* to be high (4-5 U/ml) at a range of 20-30°C, while *Pleurotus* sp. produced lower activity of MnP enzyme above and below 30°C. Huy et al. (2017) reported that MnP enzyme from *Fusarium* sp. was stable at 35°C, but became inactivated at higher temperatures. Another study on *Fusarium* sp. isolated from soil found the suitable temperature for MnP production to be 30°C, with a high rate of activity (0.35 U/ml) (Nidhi et al., 2020). *F. solani* MN201580.1 (SA17) in present study produced a substantial amount (1.26 ± 0.04 U/ml) of MnP enzyme in cultures grown under 30°C temperature. The variations in enzyme activity by *Fusarium* could be because of different strains and species of *Fusarium* used for the experimentation. However, enzyme activity was reduced to 0.10 ± 0.006 U/ml at both low (25°C) and high (40°C) temperatures, as shown in Table.17 and Figure.31-B. The enzyme was found to be active and stable at 20°C and 45°C, but decreased with increasing temperature.

Protease enzyme activity by *F. solani* MN201580.1 (SA17) showed considerable activity at 25°C to 35°C temperature range with maximum activity at 30°C. Enzyme activity rapidly increased from 188.5±1 U/ml on the 3rd day to 443±5 U/ml on the 20th day of incubation. However, the enzyme production decreased quickly to 197±1.15 U/ml on the 35th day after its peak activity. At 25°C and 35°C temperatures, the enzyme activity increased from 92±1.63 U/ml and 77±1.15 U/ml on the 5th day to 276.5±1.91 U/ml and 342±1.63 U/ml on the 25th day, respectively. After 30 days of incubation, almost equal amount of enzyme production was recorded at 25°C (154±1.63 U/ml) and 35°C (155.1±1.16 U/ml). However, the fungal strain showed less amount of enzyme activity at higher temperatures. At 40°C temperature, 28.5±1 U/ml of enzyme activity was reported on the 3rd day, which slowly increased and showed maximum activity of 62.5±1.91 U/ml on the 15th day of incubation.

According to Kamath et al. (2010), temperature plays a critical role in enzyme synthesis, with *Aspergillus* sp. exhibiting high proteolytic activity (62.5 U/ml) at 28°C after five days of incubation. *Penicillium chrysogenum* was reported to produce the highest amount of enzyme (12.79 U/ml) on the third day of incubation at 30ºC (Ikram-Ul-haq & Umber, 2006). *F. solani* MN201580.1 (SA17) in the study carried out showed a remarkable capacity to produce 188.5 ± 1 U/ml and 210.5 ± 1.91 U/ml of protease on the third and fifth day at 30ºC, respectively, which is significantly higher than other reports. The highest yield of enzyme, 443.5 ± 1.91 U/ml, was recorded on the 20th day of incubation under 30ºC temperature (Table.17 & Figure.31-C). *F. solani* MN201580.1 (SA17) appears to produce a substantial amount of protease even at higher temperatures, compared to other fungal strains. Based on these reports, the optimal temperature range for fungal proteolytic enzyme production is 28ºC to 30ºC. Fungal proteases are generally thermolabile, meaning they are not stable at high temperatures (Sharma et al., 1980). High temperatures can have adverse effects on the metabolic activities of microorganisms and inhibit their mycelial growth, according to Tunga in 1995. The protease enzyme from *F. solani* MN201580.1 (SA17) exhibited complete stability within a temperature range of 25ºC - 35ºC, but demonstrated inactivity beyond 35ºC. This may be due to the stretching and breaking of weak hydrogen bonds within the enzyme structure, resulting in denaturation of the enzyme, as described in a study by Conn et al. in 1987.

Lipase enzyme production from *F. solani* MN201580.1 (SA17) requires 30 ºC temperature as represented in Figure.31-E & Table.17. The enzyme secretion at 25°C and 35°C showed similar levels, with 28.81±0.48 U/ml and 28.85±1.53 U/ml, respectively, on the 3rd day of incubation. The peak activity was observed on the 20th day of incubation, with 48.49±1.10 U/ml and 48.64±1.32 U/ml for 25°C and 35°C, respectively. However, on the 35th day, the enzyme production decreased to 42.73 U/ml. The lowest enzyme production was observed at 40°C, with initial days showing production

between 19.47 U/ml to 24.02 U/ml. The peak activity of 30.42±0.18 U/ml was observed on the 25th day, which later reduced to 25.31±0.17 U/ml.

Based on the report, the ideal temperature range for maximum fungal lipase production is from 30ºC to 40ºC (Fu et al., 2009). However, some studies have shown that maximum production of fungal enzymes occurs at 45ºC (Liu et al., 2012; Alvarez-Macarie et al, 1999). The investigation mentioned in the report recorded the maximum lipase production at 30ºC on the 15th day after inoculation. Enzyme activity was seen to be reduced both below and above 30ºC temperature (Table.17 and Figure.31-E).

Additionally, the stability of the lipase enzyme was found to be similar to the observations of Maia et al. (2001) who reported that the thermal stability of lipase produced by *F. solani* MN201580.1 (SA17) was below 35ºC. In the present study, lipase extracted from *F. solani* MN201580.1 (SA17) was incubated, and an increase in temperature above 40ºC led to inactivation of the enzyme, and maximal stability was observed at 35ºC temperature. Lipase activity of *Rhizopus* species isolated from oil contaminated soils was examined and it revealed the optimum temperature for lipase activity to be 30 ± 1ºC (Prabhakar et al., 2012).

Table.18. Fungal biomass of *F. solani* MN201580.1 produced at different temperatures in all three culture media

Enzymes & Culture Media	Incubation period	Temperature			
		25° C	30° C	35° C	40° C
Ligninolytic, MEB (gm)	3d	0.12 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.12 ± 0.03
	5d	0.17 ± 0.03	0.15 ± 0.005	0.18 ± 0.03	0.15 ± 0.03
	10d	0.18 ± 0.02	0.19 ± 0.03	0.2 ± 0.01	0.18 ± 0.02
	15d	0.23 ± 0.03	0.23 ± 0.04	0.38 ± 0.02	0.23 ± 0.04
	20d	0.22 ± 0.01	**0.812 ± 0.01**	0.68 ± 0.01	0.37 ± 0.03
	25d	0.37 ± 0.02	0.415 ± 0.02	0.45 ± 0.03	0.35 ± 0.03
	30d	0.35 ± 0.03	0.415 ± 0.02	0.45 ± 0.02	0.23 ± 0.02
	35d	0.34 ± 0.02	0.65 ± 0.05	0.35 ± 0.03	0.23 ± 0.02
Proteolytic, Casein (gm)	3d	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.1 ± 0.001
	5d	0.14 ± 0.02	0.24 ± 0.005	0.15 ± 0.01	0.13 ± 0.01
	10d	0.16 ± 0.03	0.44 ± 0.05	0.3 ± 0.03	0.14 ± 0.02
	15d	0.28 ± 0.03	**0.47 ± 0.03**	0.31 ± 0.02	0.25 ± 0.03
	20d	0.35 ± 0.02	0.4 ± 0.005	0.38 ± 0.01	0.32 ± 0.02
	25d	0.42 ± 0.01	0.41 ± 0.01	0.32 ± 0.02	0.35 ± 0.01
	30d	0.37 ± 0.01	0.48 ± 0.01	0.3 ± 0.01	0.34 ± 0.01
	35d	0.35 ± 0.02	0.33 ± 0.01	0.28 ± 0.03	0.34 ± 0.02
Esterolytic, MSM (gm)	3d	0.58 ± 0.02	0.76 ± 0.06	0.59 ± 0.02	0.46 ± 0.03
	5d	0.69 ± 0.03	0.83 ± 0.04	0.69 ± 0.03	0.49 ± 0.03
	10d	0.75 ± 0.02	0.755 ± 0.15	0.76 ± 0.01	0.54 ± 0.02
	15d	0.93 ± 0.03	0.85 ± 0.04	0.94 ± 0.02	0.66 ± 0.04
	20d	**0.94 ± 0.01**	**0.93 ± 0.18**	**0.94 ± 0.01**	0.69 ± 0.03
	25d	0.83 ± 0.02	0.85 ± 0.07	0.84 ± 0.03	0.63 ± 0.03
	30d	0.65 ± 0.03	0.8 ± 0.15	0.65 ± 0.02	0.63 ± 0.02
	35d	0.59 ± 0.02	0.68 ± 0.08	0.65 ± 0.03	0.6 ± 0.02

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

The **esterase enzyme** activity from *F. solani* MN201580.1 (SA17) showed optimal production at 25 ºC temperature based on the data presented in Table.17 and Figure.31-D. The activity increased rapidly from 54.01±0.17 U/ml to 574.02±0.51 U/ml on the 5th day and reached its maximum of 574.02±0.51 U/ml after 20 days of incubation. However, the activity decreased to 301.19±0.84 U/ml on the 35th day. At 30 ºC, the esterase activity was lower, with 98.43±0.39 U/ml observed on the 3rd day and maximum production of only 375.63±0.63 U/ml after 25 days of incubation. At higher temperatures of 35 ºC and 40 ºC, the activity was slower and less compared to the optimum temperature of 25 ºC. The activity increased from 37.44±4.3 U/ml and 17.41±0.33 U/ml on the 3rd day to 68.36±0.33 U/ml on the 20th day and 29.55±0.5 U/ml on the 15th day at 35 ºC and 40 ºC temperatures, respectively.

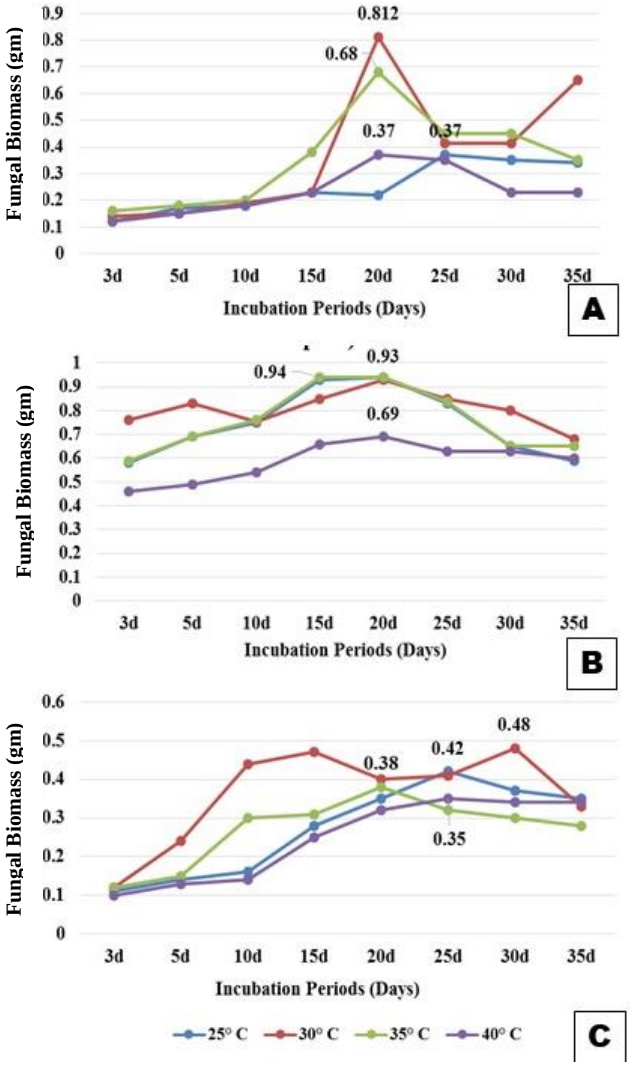

Figure.32. Fungal biomass obtained in 3 enzyme substrate media at different temperatures
A- Malt extract medium**; B-** Casein broth medium**; C-** MS Medium

The esterase activity significantly produced in culture replicates of *F. solani* MN201580.1 (SA17) incubated at 25ºC and 30ºC (Figure.31-D). According to this experiment, *F. solani* MN201580.1

(SA17) exhibited the highest esterase activity at 25ºC on the 20th day of incubation, with an activity level of 574.02 ± 0.51 U/ml. This is contrary to the findings of a previous study that reported optimal esterase production from *Fusarium* sp. at 50ºC, with complete inactivation of the enzyme occurring at temperatures above 50º C (Luo et al., 2012). However, another study on *F. sporotrichiodes* showed an increase in fungal esterase activity at 25ºC (Park & Chu, 1996). The stability of crude esterase was observed to be highest at 30ºC, with stability recorded between 15º C and 50ºC temperature ranges.

Fungal biomass in Malt extract broth medium increased gradually after a stagnant phase till 15 days of incubation at all the tested temperatures (25 ºC, 30 ºC, 35 ºC, and 40 ºC). The maximum biomass production of 0.812±0.01 gm was observed on the 20th day of incubation at 30 ºC, while at 35 ºC, the maximum growth was observed on the 20th day with a biomass production of 0.68±0.01 gm. However, the biomass reduced to 0.35±0.03 gm on the 35th day at 35 ºC. At 25 ºC and 40 ºC, the mycelial growth was slow, with the growth noted to be 0.12 gm on the 3rd day and increasing up to 0.37 gm at both temperatures. The results suggest that the optimum temperature for fungal biomass production is 30 ºC.

It seems that the fungal growth in casein broth medium was highest at 30 ºC and 35 ºC temperatures, while growth at 25 ºC and 40 ºC was comparatively slower (Table.18 & Figure.32). The fungal growth increased from 0.12±0.01 gm on the 3rd day to 0.47±0.03 gm on the 15th day of incubation at 30 ºC. In general, the fungal growth ranged from 0.1 gm to 0.12 gm on the 3rd day, which increased to 0.35±0.01 gm, 0.38±0.01 gm, and 0.42±0.01 gm on the 15th day at 40 ºC, 35 ºC, and 25 ºC temperatures, respectively. These results indicate that the optimum temperature for fungal growth in casein broth medium is around 30 ºC.

The MS broth medium showed maximum fungal growth when incubated at temperature range between 25 ºC to 35 ºC. The fungal growth was highest at 30 ºC with 0.93±0.18 gm on 20th day and reduced to 0.68±0.08 gm on 35th day. Similar amounts of fungal growth were recorded on 3rd day at 25 ºC and 35 ºC, with 0.58±0.02 gm & 0.59±0.02 gm respectively, and maximum growth recorded at 0.94±0.01 gm on 20th day at both temperatures. However, fungal growth was less at higher temperatures, with only 0.46±0.03 gm of biomass marked on 3rd day at 40 ºC and increased up to 0.69±0.03 gm on 20th day before reducing.

Although enzyme activity is not dependent on the fungal biomass, it seems that the optimal temperature for mycelial growth, as well as all enzyme activity, for *F. solani* MN201580.1 (SA17) is between 25ºC and 30ºC, based on the results obtained in this investigation. A research study on *F. oxysporium* supported the substantial effect of incubation temperature on the growth, the species showed highest growth at 30ºC incubation temperature. Likewise, *F. solani* MN201580.1 (SA17) cultures inoculated in MEB and Casein broth medium evidenced maximum growth at 30ºC and the

growth rate declined in high temperature degree. In both the culture medium, highest fungal biomass was observed on five days earlier to the day on which maximum enzyme activity recorded.

Ligninolytic and proteolytic enzyme activity were highest on 25^{th} & 20^{th} day, whereas, substantial fungal growth was observed on 20^{th} & 15^{th} day respectively (Table.17 & 18). *F. solani* MN201580.1 (SA17) cultures grown in MS broth medium showed maximum mycelial growth on 15^{th} and 20^{th} incubation day, which correlates with the highest levels of enzyme activity observed at 25ºC and 30ºC temperature. The maximum mycelial growth was observed to be similar at 25ºC, 30ºC, and 35ºC. However, the enzyme activity decreased with the increase in temperature beyond 30ºC, and the fungal biomass also decreased after 35ºC temperature. These experiments revealed significant correlation between fungal growth and enzyme production.

These findings underscore the importance of identifying the optimal incubation temperature for each strain of fungi, as this can have a significant impact on the production and activity of polyethylene degrading enzymes.

Determination of optimum pH for enzyme activities

The optimal pH for fungal growth and enzyme production varies among different fungal strains and enzymes. Some fungal species prefer acidic environments and grow best in pH ranges between 4 and 6, while others prefer alkaline conditions and grow best in pH ranges between 8 and 9. The optimal pH for enzyme activity also varies depending on the type of enzyme. For example, fungal lipases usually have an optimal pH range between 4 and 6, while fungal proteases typically have an optimal pH range between 7 and 9.

In this experiment, it was found that fungal enzymes were stable at pH ranging from 4 to 8. The capability of fungal growth to produce these enzymes at different pH levels ranging from 4-14 pH of culture media was examined. It was observed that all enzymes showed maximum production in culture media at pH 8, except for the esterase enzyme, which also had considerable production at pH 8.

Laccase enzyme was slow and steady until the 15th day across all pH levels. However, at pH 6, 7, and 8, the enzyme activity showed an exponential increase with maximum activity on the 25th day for pH 6 and on the 20th day for pH 7. Across the four pH levels tested, the culture replicates showed 0.59±0.06 U/ml activity on the 3rd day, which peaked at 0.89±0.1 U/ml on the 20th day before declining to 0.55±0.09 U/ml on the 35th day. On the 3rd day, the highest enzyme activity of 1.28±0.5 U/ml was observed at pH 6, while the lowest activity of 0.57±0.05 U/ml was observed at pH 7. The enzyme activity peaked on the 25th day with 2.25±1.78 U/ml of enzyme production at pH 6, whereas at pH 7, the activity peaked on the 15th day and showed 1.55±0.11 U/ml of enzyme production.

Laccase enzyme production is pH-dependent, with pH 8 being the optimal pH for enzyme production. The enzyme production increased from 1.04±0.06 U/ml on 3rd day to 14.50±0.41 U/ml on 25th day

at pH 8, which then decreased to 11.07±0.06 U/ml on 35th day. At pH 9 and 10, the enzyme production was lower, with 1.04±0.06 U/ml and 0.99±0.05 U/ml recorded on 3rd day, respectively. However, the enzyme production gradually increased to 2.82±0.02 U/ml (5th day) and 3.29±0.59 U/ml (15th day) at pH 9 and 10, respectively, which then slightly dropped in later days. At higher pH levels of 12 and 14, the maximum enzyme production recorded was 2.70±0.04 U/ml (20th day) and 2.03±0.02 U/ml (15th day), respectively.

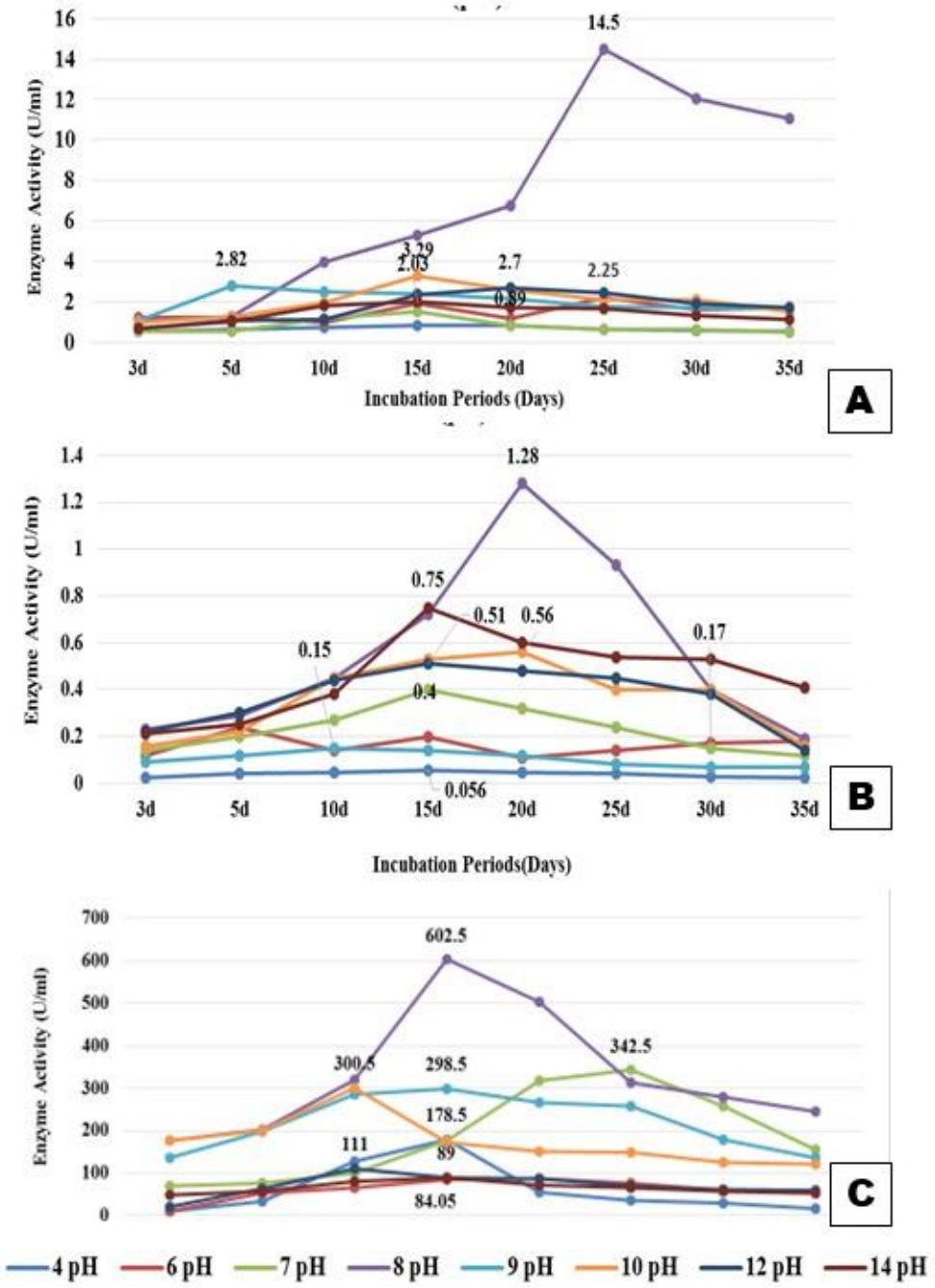

Figure.33. Quantitative analysis of enzyme activities by *F. solani* at different pH levels
A- Laccase activity; **B-** MnP activity; **C-** Protease activity

Table.19. Enzyme production by *F. solani* MN201580.1 in different pH level of all three culture media

Enzyme Activity	Incubation period	pH Level							
		4	6	7	8	9	10	12	14
Laccase (U/ml)	3d	0.59 ± 0.06	1.28 ± 0.5	0.57 ± 0.05	1.04 ± 0.06	1.10 ± 0.02	0.99 ± 0.05	0.72 ± 0.029	0.71 ± 0.02
	5d	0.65 ± 0.16	1.27 ± 0.46	0.59 ± 0.03	1.31 ± 0.07	2.82 ± 0.02	1.32 ± 0.08	1.13 ± 0.02	1.04 ± 0.02
	10d	0.77 ± 0.09	0.99 ± 0.35	1.22 ± 0.19	4 ± 0.33	2.52 ± 0.1	2.01 ± 0.89	1.16 ± 0.02	1.85 ± 0.02
	15d	0.86 ± 0.05	1.95 ± 1.42	1.55 ± 0.11	5.32 ± 0.76	2.44 ± 0.03	3.29 ± 0.59	2.40 ± 0.02	2.03 ± 0.02
	20d	0.89 ± 0.1	1.22 ± 0.04	0.89 ± 0.1	6.76 ± 0.65	2.16 ± 0.01	2.62 ± 0.14	2.70 ± 0.04	1.75 ± 0.04
	25d	0.69 ± 0.07	2.25 ± 1.78	0.69 ± 0.07	**14.50 ± 0.41**	1.79 ± 0.04	2.15 ± 0.06	2.47 ± 0.04	1.71 ± 0.04
	30d	0.62 ± 0.06	1.63 ± 0.33	0.69 ± 0.09	12.05 ± 0.23	1.75 ± 0.015	2.12 ± 0.02	1.93 ± 0.04	1.35 ± 0.02
	35d	0.55 ± 0.09	1.75 ± 0.22	0.51 ± 0.04	11.07 ± 0.06	1.71 ± 0.02	1.56 ± 0.05	1.73 ± 0.02	1.14 ± 0.02
MnP (U/ml)	3d	0.02 ± 0.005	0.12 ± 0.02	0.14 ± 0.003	0.23 ± 0.005	0.09 ± 0.003	0.16 ± 0.01	0.22 ± 0.005	0.21 ± 0.004
	5d	0.04 ± 0.01	0.24 ± 0.003	0.2 ± 0.007	0.29 ±0.01	0.12 ± 0.008	0.22 ± 0.009	0.3 ± 0.01	0.25 ± 0.005
	10d	0.04 ± 0.005	0.14 ± 0.03	0.27 ± 0.003	0.45 ± 0.01	0.15 ± 0.007	0.45 ± 0.01	0.44 ± 0.01	0.38 ± 0.007
	15d	0.05 ± 0.005	0.2 ± 0.002	0.4 ± 0.007	0.72 ± 0.05	0.14 ± 0.01	0.53 ± 0.01	0.75 ± 0.002	0.75 ± 0.005
	20d	0.04 ± 0.008	0.11 ± 0.02	0.32 ± 0.01	**1.28 ± 0.01**	0.12 ± 0.007	0.56 ± 0.01	0.76 ± 0.01	0.6 ± 0.01
	25d	0.04 ± 0.007	0.14 ± 0.006	0.24 ± 0.005	0.93 ± 0.08	0.08 ± 0.005	0.40 ± 0.01	0.97 ± 0.01	0.54 ± 0.008
	30d	0.02 ± 0.007	0.17 ± 0.003	0.15 ± 0.002	0.4 ± 0.03	0.07 ± 0.007	0.4 ± 0.03	0.38 ± 0.01	0.53 ± 0.006
	35d	0.02 ± 0.003	0.18 ± 0.06	0.12 ± 0.003	0.19 ± 0.07	0.07 ± 0.003	0.16 ± 0.06	0.14 ± 0.01	0.41 ± 0.01
Protease (U/ml)	3d	11.25 ± 0.34	10.65 ± 0.3	70.25 ± 0.3	177 ± 1.15	135.5 ± 1.91	176 ± 1.63	21.5 ± 1	49.5 ± 1
	5d	33.3 ± 0.2	52.3 ± 0.34	76.6 ± 0.95	202.5 ± 1	197.5 ± 1	197.5 ± 1.03	64.5 ± 1	56.5 ± 1
	10d	128.2 ± 0.23	65.5 ± 1.91	99.6 ± 0.16	319.5 ± 1	285 ± 1.15	300.5 ± 1	111 ± 1.15	81.5 ± 1.91
	15d	178.5 ± 0.97	84.05 ± 0.1	176.5 ± 0.97	**602.5 ± 1**	298.5 ± 1	172 ± 1.63	89.5 ± 1.91	89 ± 1.15
	20d	54.65 ± 0.91	86.25 ± 0.19	317 ± 1.15	502 ± 1.63	165.5 ± 1	150.5 ± 0.97	87 ± 1.15	72.1 ± 0.2
	25d	36.65 ± 0.91	76.05 ± 0.1	342 ± 1.63	405 ± 1.1	257 ± 1.15	148.5 ± 1	70 ± 1.63	65 ± 1.15
	30d	28.65 ± 0.91	58.55 ± 0.97	158.5 ± 1	279 ± 1.15	179.5 ± 1	126.05 ± 1.63	61 ± 1.15	57 ± 1.15
	35d	16.65 ± 0.91	52.2 ± 0.28	156.4 ± 0.34	246 ± 1.63	137 ± 1.15	120.5 ± 1	59 ± 1.15	54.05 ± 0.1
Esterase U/ml)	3d	37.44 ± 3.04	46.63 ± 1.37	35 ± 0.13	19.79 ± 0.58	13.10 ± 0.35	16.73 ± 0.78	19.74 ± 0.56	16.16 ± 0.35
	5d	44.25 ± 0.48	56.16 ± 1.66	54.35 ± 0.55	34.77 ± 0.25	28.08 ± 0.59	24.67 ± 0.59	34.94 ± 0.46	120.55 ± 0.39
	10d	97.97 ± 0.39	63.37 ± 2.70	338.52 ± 3.06	188.75 ± 0.41	34.26 ± 0.27	54.01 ± 0.70	187.84 ± 0.69	135.1 ± 0.58
	15d	147.73 ± 0.43	83.85 ± 3.38	517.29 ± 0.59	295.07 ± 0.54	136.61 ± 0.27	154.76 ± 1.04	238.50 ± 0.27	188.75 ± 0.41
	20d	129.86 ± 0.35	64.05 ± 2.41	**599.44 ± 0.71**	419.2 ± 0.35	279.69 ± 0.51	226.47 ± 0.51	268.17 ± 0.50	170.9 ± 0.27
	25d	162.54 ± 0.59	57.75 ± 1.38	389.36 ± 1.08	**525.35 ± 0.17**	271.52 ± 0.46	192.32 ± 0.74	211.89 ± 0.55	155.39 ± 0.35
	30d	205.43 ± 0.35	49.35 ± 3.41	350.15 ± 2.42	229.88 ± 0.17	168.32 ± 0.41	166.11 ± 0.21	193.12 ± 0.46	136.84 ± 0.21
	35d	204.29 ± 0.5	39.82 ± 1.32	301.7 ± 1.19	166.11 ± 0.215	72.73 ± 0.41	134.91 ± 0.73	165.88 ± 0.41	127.87 ± 0.27
Lipase (U/ml)	3d	41.14 ± 0.49	21.47 ± 0.16	27.66 ± 1.98	102.85 ± 0.39	59.33 ± 0.41	102.7 ± 0.35	41.71 ± 0.16	54.38 ± 0.31
	5d	81.8 ± 1.03	21.9 ± 0.51	37.66 ± 1.87	112.66 ± 0.33	71.19 ± 0.19	112.6 ± 0.44	64.95 ± 0.13	65.38 ± 0.27
	10d	83.26 ± 0.7	29.45 ± 2.39	45.44 ± 1.36	126.04 ± 0.23	92.98 ± 0.19	113.3 ± 0.35	77.7 ± 0.31	78.26 ± 0.29
	15d	90.1 ± 0.68	35.31 ± 1.35	44.93 ± 0.57	137.92 ± 0.42	64.89 ± 0.31	99.7 ± 0.3	83.6 ± 0.27	79.7 ± 0.37
	20d	118.22 ± 0.47	41.75 ± 1.21	48.68 ± 1.39	**210.2 ± 0.51**	62.73 ± 0.41	79.66 ± 0.46	84.5 ± 0.18	77.28 ± 0.26
	25d	85.23 ± 0.22	38.87 ± 0.96	44.51 ± 1.21	136.03 ± 0.31	54.55 ± 0.56	68.03 ± 0.66	64.96 ± 0.27	67.2 ± 0.31
	30d	79.85 ± 0.59	35.95 ± 0.77	43.94 ± 1.33	128.79 ± 0.12	47.77 ± 0.64	65.53 ± 0.39	65.3 ± 0.26	52.4 ± 0.31
	35d	73.83 ± 0.27	33.98 ± 0.71	42.73 ± 0.68	106.75 ± 0.53	31.67 ± 0.44	49.66 ± 0.55	62.35 ± 0.17	38.83 ± 0.37

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

F. solani MN201580.1 (SA17) is observed to produce significant amounts of ligninolytic enzymes in basic medium, with maximum laccase activity of 14.50 ± 0.41 U/ml at pH 8 after 25 days of incubation. The activity of laccase gradually decreased beyond pH 8 of the growth medium as presented in Figure.33-A and Table.19. In contrast, Chhaya and Gupte (2013) found that *F. incarnatum* strain produces laccase enzymes most effectively in the pH range of 3 to 5, with maximum release (471.02 U/g) at pH 5.

The enzyme remains active in pH range 4 to 9, with optimum stability at pH 8, but becomes completely inactivated above pH 9. Similarly, *Fomes fomentarius* strain showed optimum stability for enzyme activity at pH 8 (Solcany et al., 2016). Kumar et al. (2012) noted that laccase is typically active in the pH middle layer. Unfavorable pH levels can limit mycelial growth and production by reducing the availability of the substrate, and that pH changes can alter the structure of the enzyme, as explained by Shulter and Kargi (2000).

MnP enzyme production started to increase significantly after 5 days of inoculation and reached a maximum level after 15-20 days, followed by a reduction in production. The enzyme showed the highest production in culture replicates grown in pH 8 of the growth medium and the lowest activity at pH 4. In the replicates with pH 4, only a slight increase in enzyme activity was observed from 0.02±0.005 U/ml on day 3 to 0.05±0.005 U/ml on day 15. On the other hand, in pH 6 replicates, the enzyme activity was 0.12±0.02 U/ml on day 3 and reached a maximum of 0.24±0.003 U/ml on day 5. At neutral pH, MnP enzyme increased from 0.14±0.003 U/ml on 3rd day to 0.27±0.003 U/ml on 10th day, peaked at 0.4±0.007 U/ml on 15th day and reduced to 0.12±0.003 U/ml on 35th day of incubation. In slightly basic medium with pH 8 significant amount of enzyme production recorded, on 3rd day 0.23±0.005 U/ml of enzyme, which risen up to maximum activity with 1.28±0.01 U/ml on 20th day and further declined to 0.19±0.07 U/ml on 35th day. In basic medium with pH 9 & 10, 0.09±0.003 U/ml & 0.16±0.01 U/ml of enzyme released on 3rd day with peak activity 0.15±0.007 U/ml (10th day) & 0.56±0.01 U/ml (20th day) correspondingly. However, with higher pH level the enzyme activity seemed to be increased, 0.22±0.005 U/ml and 0.21±0.004 U/ml enzyme production recorded on 3rd day which increased up to 0.97±0.01 U/ml (25th day) and 0.75±0.005 U/ml (15th day) in pH 12 & 14 respectively.

The data presented in Figure.33-B and Table.19 shows an exponential increase in the production of MnP enzyme from pH 4 to 8, with maximum production of 1.28 ± 0.01 U/ml at pH 8 on the 20th day of incubation. The enzyme was reduced in the pH 9 culture medium, but increased again with increasing pH up to 0.97 ± 0.01 U/ml, although the amount is less than that received at pH 8. The stability of MnP crude enzyme was examined and found to be similar to laccase enzyme stability. However, Huy et al. (2017) detected high activity of MnP by *Fusarium* species in acidic medium with

a pH around 4, and it was stable between pH 3 to 5. Nidhi et al. (2020) quantified the MnP enzyme produced by an unknown species of *Fusarium* and found that 0.380 U/ml amount of activity was obtained at pH 6 in 4 days of fermentation period.

Protease enzyme activity was found to be optimal in neutral to slightly basic culture medium as clearly depicted in Figure.33-C and Table.19. The highest enzyme activity was found in the pH 8 medium, with enzyme levels of 177±1.15 U/ml and 202.5±1 U/ml on the third and fifth day, respectively. The maximum enzyme activity was achieved on the 15th day, with an activity level of 602.5±1 U/ml, which then declined to 246±1.63 U/ml on the 35th day. In contrast, in basic media with a pH of 4 and 6, enzyme activities of only 11.25±0.34 U/ml and 10.65±0.3 U/ml were observed on the third day, respectively. These enzyme levels only increased up to 178.5±0.97 U/ml (15 days) and 86.25±0.19 U/ml (20 days), respectively.

In the neutral growth medium with pH 7, the enzyme activity was found to be 70.25±0.3 U/ml on the 3rd day and increased significantly to 342±1.63 U/ml on the 25th day. However, the enzyme activity appeared to decrease with an increase in pH level, and equal amounts of enzyme production were observed in the pH 9 and 10 culture medium. The enzyme activity increased from 135.5±1.91 U/ml and 176±1.63 U/ml on the 3rd day to 298.5±1 U/ml (15 days) and 300.5±1 U/ml in the pH 9 and 10 culture medium, respectively. After reaching its peak activity, the enzyme activity gradually decreased to 257±1.15 U/ml (25th day) and 172±1.63 U/ml (15th day) and further reduced to 137±1.15 U/ml and 120.5±1 U/ml of enzyme activity on the 35th day in pH 9 and 10, respectively. When the culture was grown in higher pH levels of 12 and 14, the enzyme activity was recorded as 21.5±1 U/ml and 49.5±1 U/ml on the 3rd day and increased only to 111±1.15 U/ml (10th day) and 89±1.15 U/ml (15th day), respectively.

Growth media pH of the strongly encourage many enzymatic processes and is directly linked with cell growth and product production (Ellaiah et al., 2002). *F. graminearum* was found to produce only acidic protease with an optimum pH of 5 when grown in a casein medium with a good amount of glucose. However, when grown in a medium with limited glucose, the fungus was able to produce both acidic and alkaline proteases (Griffen et al., 1997). In the present investigation, *F. solani* MN201580.1 (SA17) was found to secrete neutral to alkaline proteases in casein broth medium with a lower amount of glucose (Table.19 & Figure.28). Many *Fusarium* species, such as *F. sambucinum*, *F. graminearum*, and *F. oxysporum*, are capable of producing alkaline protease in different growth media (Kladnitskaya et al., 1994; McKay, 1992). Meanwhile, Urbanek and Yirdaw (1978) found that *F. culmorum*, *F. avenaceum*, and *F. oxysporum* species were able to produce acidic protease when cultured in acidic growth medium.

Previously reported studies revealed fungal protease require optimum pH level 7 for maximum yield, *A. niger* and *F. oxysporium* both fungi reported for maximum protease production in neutral culture medium (Paranthaman et al., 2009; Ali & Vidhale, 2013). A soil fungus *F. graminearum* showed maximum enzyme productivity 4.85 U/ml in culture medium with pH level 6.6 on 5th day of incubation (Kumari et al., 2010). These results indicated the potentiality of *F. solani* MN201580.1 (SA17) strain to produce most stable enzyme with pH stability range 4 to 12 and exhibited high enzyme activity in neutral to slightly alkaline medium. The activity was adversely affected at a pH level below & above pH 8. The optimum protease activity of 602.5 ± 1 U/ml was observed on 15th day of incubation. However, the optimum pH recorded as 8, but cultures grown in medium with 7 pH also indicated substantial activity (342 ± 1.63 U/ml) of enzyme on 25th day. Which explains that pH 7 & 8 both levels are suitable for significant protease enzyme activity.

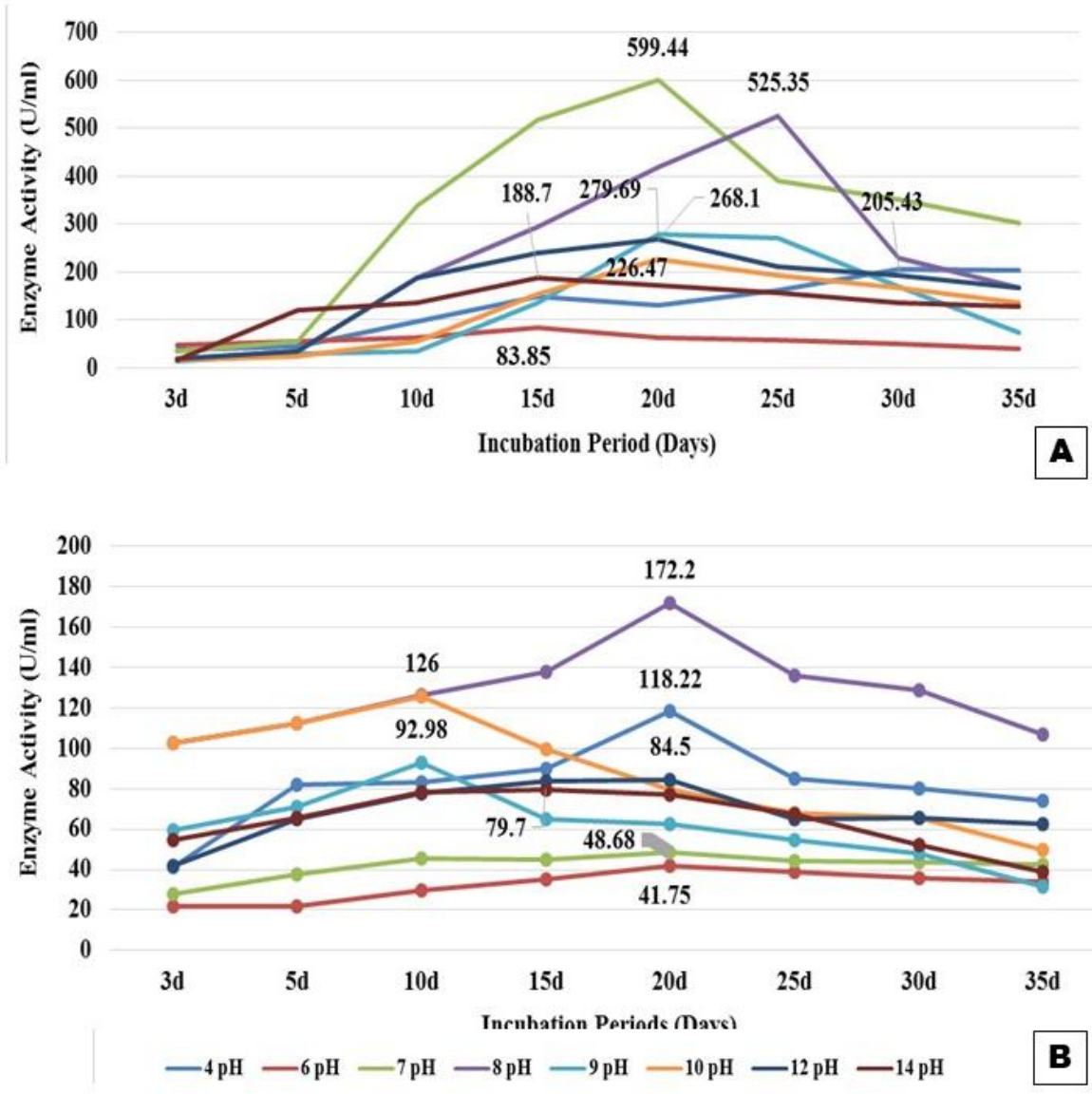

Figure.34. Quantitative analysis of enzyme activities by *F. solani* at different temperatures: A- Esterase activity; **B-** Lipase activity

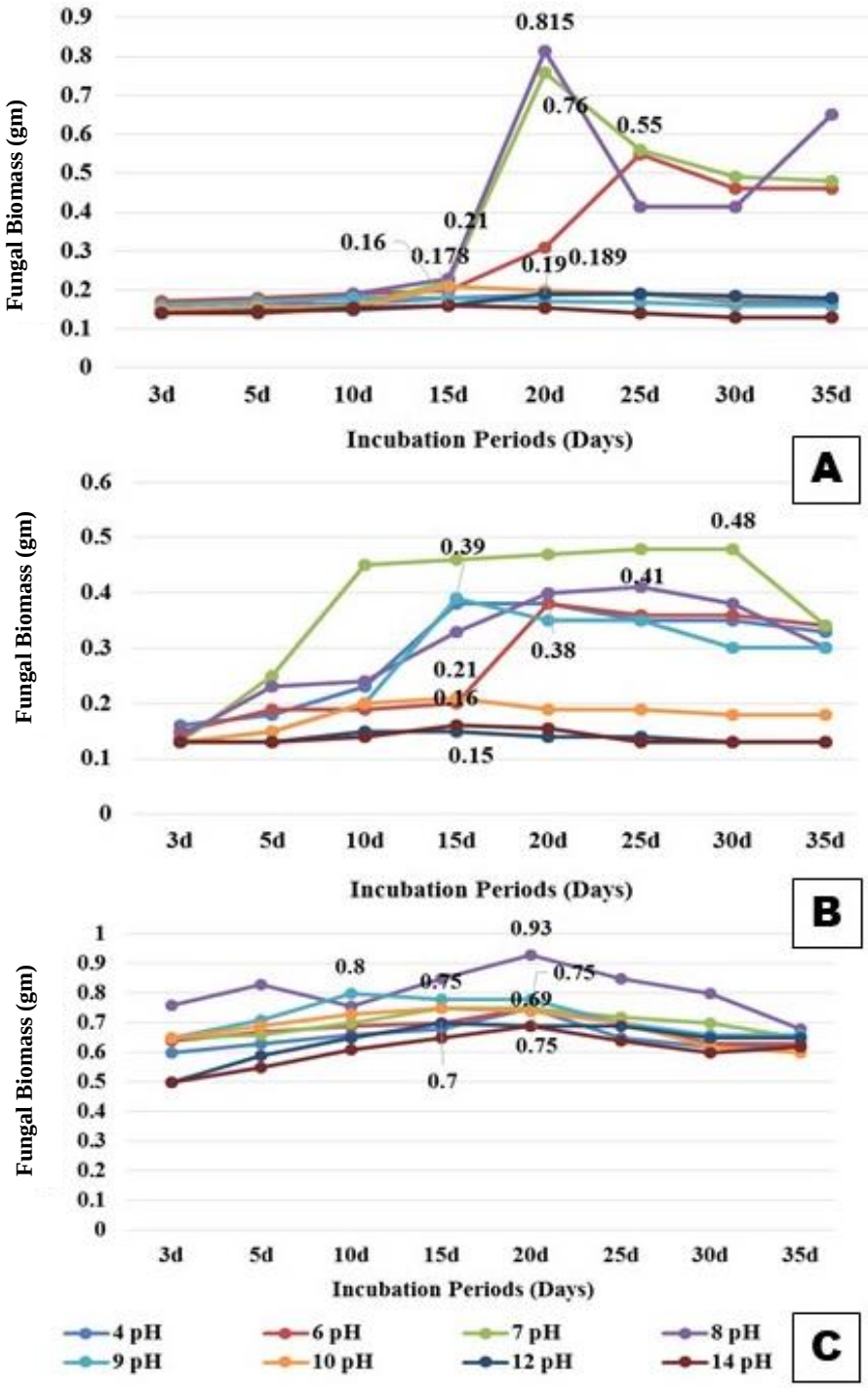

Figure.35. Fungal biomass obtained in 3 enzyme substrate media at different pH levels
A- Malt extract medium**; B-** Casein broth medium**; C**- MS Medium

The **esterase enzyme** demonstrated an exponential increase in pH levels 7 and 8, with 35±0.13 U/ml and 19.79±0.58 U/ml noted on the 3rd day, which quickly rose to 599.44±0.71 U/ml (20 days) and 525.35±0.17 U/ml (25 days) and decreased to 301.7±1.19 U/ml and 166.11±0.215 U/ml on the 35th day, as shown in Figure.29 and Table.19. In contrast, the acidic broth medium with pH 4 and 6 displayed more enzyme activity than other pH ranges in the initial days of incubation. 37.44±3.04 U/ml and 46.63±1.37 U/ml of enzyme activity were recorded after 3 days and slowly peaked at 205.43±0.35

U/ml (30 days) and 83.85±3.38 U/ml (15 days) in pH levels 4 and 6, respectively. Esterase activity decreased with higher pH levels, with 13.10±0.35 U/ml (pH 9), 16.73±0.78 U/ml (pH 10), 19.74±0.56 U/ml (12 pH), and 16.16±0.35 U/ml (pH 14) recorded after 3 days of incubation. Enzyme production slowly peaked at 279.69±0.51 U/ml (20th day), 226.47±0.51 U/ml (20th day), 268.17±0.50 U/ml (20th day), and 188.75±0.41 U/ml (15th day) in pH levels 9, 10, 12, and 14, respectively.

The pH level of the growth medium has a significant impact on the stability of enzymes, as noted by Ueda et al. (1982). The production of esterase enzyme from *Lantinus tigrinus* was found to be highly influenced by pH, with the species preferring neutral to acidic conditions for production, according to Tahir et al. (2013). In contrast, the esterase produced by *F. solani* MN201580.1 (SA17) was most active in a neutral to slightly basic medium. As shown in Figure.34-A, a high yield of esterase (599.44 ± 0.71 U/ml) was recorded at pH level 7 on the 20th day of incubation, and almost the same amount (525.35 ± 0.17 U/ml) of esterase was produced in a culture medium with a pH of 8 on the 25th day of incubation. This indicates that in a slightly basic culture medium, the esterase activity was delayed for five days, but the production was still somewhat equivalent to that in pH 7. The enzyme was found to be fully active in the pH range of 4 to 9 and diminished in highly basic pH. The potential of *Fusarium* to produce esterase enzyme under different parameters is demonstrated by the present investigation.

Lipase enzyme activity found to be optimum in basic culture medium as seen in Figure.34-B and Table.19. At pH level 8, the production slowly increased from 102.85±0.39 U/ml on 3rd day to 126.04±0.23 U/ml on 10th day and quickly lifted to 210.2±0.51 U/ml after 20 days of incubation and dropped at 16.75±0.53 U/ml on 35th day. In contrast to that, the activity slowly reached up to 92.98±0.19 U/ml and 113.3±0.35 U/ml after 10 days and instantly decreased to 54.55±0.56 U/ml & 68.03±0.66 U/ml on 25th day which reduced further more in pH 9 & 10 respectively. In higher pH 12 and 14 levels, the activity recorded as 41.71±0.16 U/ml and 54.38±0.31 U/ml on 3rd day of incubation which increased only up to 84.5±0.18 U/ml (20 days) and 79.7±0.37 U/ml (15 days), respectively. Whereas in acidic medium, 41.14±0.49 U/ml, 21.47±0.16 U/ml & 27.66±1.98 U/ml amount of enzyme production recorded on 3rd day in pH 4, 6 & 7 respectively. The activity peaked at 118.22±0.47 U/ml, 41.75±1.21 U/ml and 48.68±1.39 U/ml after 20 days of incubation in pH 4, 6 & 7 and which reduced to 73.83±0.27 U/ml, 33.98±0.71 U/ml & 42.73±0.68 U/ml on 35th day, respectively.

Fungal lipase is reported to be produced at high level in alkaline medium than acidic medium. Lipase from *F. oxysporum* was reported to be active in pH range 6 to 7, and exhibited maximum activity (60 U/ml) at pH 7, the enzyme was more stable in alkaline pH (Prazeres et al., 2006). Mahmoud et al. (2015) stated optimum pH range 7 to 9 for lipase production from *A. terreus*. Although *F. solani* MN201580.1 (SA17) was able to grow in wide range of pH, it showed highest lipase production 210.2 ± 0.51 U/ml at pH level 8 and indicated stability between 5 to 12. The lipase activity discovered to be

degreased below and beyond 8 pH level of the culture medium as seen in Table.19 & Figure.35-C. Another species of *Fusarium*, *F. globulosum* produced highest lipase 9.5 U/ml in four days at 7 pH (Gulati et al., 2005), whereas in present study the species of *Fusarium* was able to release 37.66 ± 1.87 U/ml in five days at the same pH level. These findings suggest that *F. solani* MN201580.1 (SA17) has the potential to produce significant amounts of lipase, particularly in slightly alkaline environments.

According to the results, in Malt broth medium, fungal biomass remained stable until day 15, and then rapidly increased on the 20th day of incubation with reduced growth after that time. In cultures with pH levels of 7 and 8, significant mycelial growth was observed, with 0.15 gm of biomass recorded on the 3rd day, which increased to 0.76±0.01 gm and 0.815±0.01 gm on the 20th day in both pH levels. In acidic medium, fungal growth was recorded as 0.15±0.01 gm and 0.17±0.02 gm on the 3rd day, and reached 0.19±0.02 gm and 0.55±0.02 gm on the 25th day in pH 4 and 6, respectively. In basic pH levels of 9, 10, 12, and 15, maximum growth was observed as 0.169±0.02 gm (25 days), 0.21±0.02 gm (15 days), 0.19±0.02 gm (20 days), and 0.16±0.02 gm (15 days), respectively.

Casein broth medium with a neutral pH of 7 showed the highest fungal biomass, with 0.13±0.01 gm of biomass detected on the 3rd day, rapidly increasing to 0.48±0.01 gm after 25 days of incubation. Although enzyme activity was found to be high at pH 8, the fungal biomass was observed to be lower than that at the neutral pH, with 0.14±0.02 gm of biomass detected after 3 days and a slow increase to 0.41±0.02 gm on the 25th day of incubation. In the acidic growth medium with pH 4 and 6, 0.16±0.01 gm and 0.15±0.02 gm of fungal biomass were detected on the 3rd day, respectively, which reached up to 0.38±0.01 gm after 20 days of incubation in both pH levels. Fungal biomass of 0.13±0.02 gm was recorded on the 3rd day when grown in pH levels ranging from 9 to 14, which increased to 0.39±0.02 gm (pH 9), 0.21±0.02 gm (pH 10), 0.15±0.01 gm (pH 12), and 0.16±0.02 gm (pH 14) on the 15th day of incubation.

The fungal growth in MS broth culture media appeared to be similar across all pH levels, as shown in Table.20 and Figure.35. However, there was a slight increase in biomass at pH 8, with fungal biomass increasing from 0.76±0.06 gm on the 3rd day to 0.93±0.18 gm after 20 days of incubation. In acidic media, biomass was observed to be between 0.6 to 0.65 gm on the third day, which reached a maximum of 0.75 gm after 20 days of incubation in pH 4, 6, and 7. On the third day, biomass of 0.65±0.02 gm was noted, which increased to 0.78±0.02 gm and 0.75±0.01 gm on the 15th day in pH levels 9 and 10, respectively. Meanwhile, in pH 12 and 14, only 0.5 gm of biomass was recorded on the third day, and the maximum growth observed was 0.69 gm on the 20th day, decreasing to 0.65±0.02 gm and 0.62±0.04 gm on the 35th day in both pH levels.

Table.20. Fungal biomass of *F. solani* MN201580.1 produced in different pH level of all three culture media

Enzymes & Culture Media	Incubation period	pH Level							
		4	**6**	**7**	**8**	**9**	**10**	**12**	**14**
Ligninolytic, MEB (gm)	**3d**	0.15 ± 0.01	0.17 ± 0.02	0.15 ± 0.01	0.15±0.02	0.16 ± 0.02	0.15 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
	5d	0.16 ± 0.01	0.178 ± 0.01	0.151 ± 0.03	0.155±0.005	0.17 ± 0.02	0.158 ± 0.01	0.145 ± 0.02	0.14 ± 0.01
	10d	0.165 ± 0.02	0.19 ± 0.03	0.16 ± 0.01	0.19±0.03	0.18 ± 0.01	0.155 ± 0.02	0.15 ± 0.02	0.155 ± 0.02
	15d	0.18 ± 0.02	0.2 ± 0.01	0.22 ± 0.04	0.23±0.04	0.178 ± 0.02	0.21 ± 0.02	0.16 ± 0.01	0.16 ± 0.02
	20d	0.189 ± 0.01	0.31 ± 0.01	0.76 ± 0.01	**0.815±0.01**	0.171 ± 0.01	0.2 ± 0.03	0.19 ± 0.02	0.155 ± 0.03
	25d	0.19 ± 0.02	0.55 ± 0.02	0.56 ± 0.07	0.415±0.02	0.169 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.14 ± 0.02
	30d	0.17 ± 0.02	0.46 ± 0.03	0.49 ± 0.02	0.415±0.02	0.16 ± 0.01	0.18 ± 0.02	0.186 ± 0.02	0.13 ± 0.01
	35d	0.17 ± 0.02	0.46 ± 0.02	0.48 ± 0.02	0.65±0.05	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.13 ± 0.02
Proteolytic, Casein (gm)	**3d**	0.16 ± 0.01	0.15 ± 0.02	0.13 ± 0.01	0.14 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.02
	5d	0.18 ± 0.01	0.19 ± 0.01	0.25 ± 0.005	0.23 ± 0.005	0.15 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	0.13 ± 0.01
	10d	0.23 ± 0.02	0.19 ± 0.03	0.45 ± 0.05	0.24 ± 0.03	0.2 ± 0.01	0.2 ± 0.02	0.15 ± 0.02	0.14 ± 0.02
	15d	0.38 ± 0.02	0.2 ± 0.01	0.46 ± 0.03	0.33 ± 0.04	0.39 ± 0.02	0.21 ± 0.02	0.15 ± 0.01	0.16 ± 0.02
	20d	0.38 ± 0.01	0.38 ± 0.01	0.47 ± 0.005	0.4 ± 0.01	0.35 ± 0.01	0.19 ± 0.03	0.14 ± 0.02	0.155 ± 0.03
	25d	0.35 ± 0.02	0.36 ± 0.02	**0.48 ± 0.01**	0.41 ± 0.02	0.35 ± 0.02	0.19 ± 0.02	0.14 ± 0.02	0.13 ± 0.02
	30d	0.35 ± 0.02	0.36 ± 0.03	0.48 ± 0.01	0.38 ± 0.02	0.3 ± 0.01	0.18 ± 0.02	0.13 ± 0.02	0.13 ± 0.01
	35d	0.33 ± 0.02	0.34 ± 0.02	0.34 ± 0.01	0.3 ± 0.05	0.3 ± 0.02	0.18 ± 0.02	0.13 ± 0.02	0.13 ± 0.02
Esterolytic, MSM (gm)	**3d**	0.6 ± 0.03	0.64 ± 0.02	0.65 ± 0.03	0.76±0.06	0.65 ± 0.02	0.65 ± 0.02	0.5 ± 0.01	0.5 ± 0.02
	5d	0.63 ± 0.03	0.67 ± 0.01	0.66 ± 0.02	0.83±0.04	0.71 ± 0.03	0.69 ± 0.03	0.59 ± 0.02	0.55 ± 0.03
	10d	0.66 ± 0.02	0.69 ± 0.02	0.7 ± 0.03	0.755±0.15	0.8 ± 0.02	0.73 ± 0.02	0.65 ± 0.01	0.61 ± 0.02
	15d	0.68 ± 0.04	0.7 ± 0.01	0.75 ± 0.02	0.85±0.04	0.78 ± 0.01	0.75 ± 0.01	0.7 ± 0.02	0.65 ± 0.03
	20d	0.75 ± 0.02	0.75 ± 0.03	0.75 ± 0.03	**0.93±0.18**	0.78 ± 0.02	0.74 ± 0.02	0.69 ± 0.02	0.69 ± 0.01
	25d	0.65 ± 0.02	0.7 ± 0.02	0.72 ± 0.02	0.85±0.07	0.7 ± 0.03	0.7 ± 0.01	0.69 ± 0.02	0.64 ± 0.02
	30d	0.62 ± 0.03	0.63 ± 0.02	0.7 ± 0.03	0.8±0.15	0.66 ± 0.02	0.62 ± 0.02	0.65 ± 0.03	0.6 ± 0.03
	35d	0.62 ± 0.04	0.63 ± 0.03	0.65 ± 0.02	0.68±0.08	0.66 ± 0.03	0.6 ± 0.01	0.65 ± 0.02	0.62 ± 0.04

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

Ligninolytic enzyme activity was high in slightly basic culture medium and fungal biomass (0.815±0.01 gm) in MEB medium with pH 8 was recorded to be maximum at that pH level. Fungal cultures grown in casein medium with neutral pH level 7 (0.48 ± 0.01 gm) showed maximum mycelial growth compared to pH 8 (0.41 ± 0.02 gm) as displayed in Figure.33-A. *F. globulosum* showed maximum lipase activity at 7 pH in four days and 0.015 gm fungal biomass at the same time and condition (Gulati et al., 2005), in the case of *F. solani* MN201580.1 (SA17) 0.66 ± 0.02 gm biomass in MS medium was recorded in five days at the same pH level (Table.20). In all three-growth medium, fungal biomass exhibited strong relation with pH range as the biomass observed to be reduced under unfavorable pH level. It is noted that fungal biomass tends to decrease under unfavorable pH conditions, indicating the importance of maintaining appropriate pH levels in fungal culture media for optimal growth and enzyme production.

The pH of the growth medium can have a significant impact on both fungal growth and enzyme production, and it is important to carefully control this parameter in order to maximize enzyme yield and activity.

The optimized condition required for the fungal species studied to produce the maximum enzyme activities are identified as follows. The identified optimized condition was selected for the further experimentation of polyethylene degradation.

Table.21. Optimized condition for the enhanced enzyme production to degrade polyethylene material

Inoculum Size	Incubation Period	Temperature	pH
Single disc	20^{th} day	30ºC	8 (Basic medium)

Optimization of enzyme extraction methodology for solid state medium under simulated optimized conditions:

The solid-state fermentation technique holds great importance as an efficient biotechnological process. The present study on *F. solani* MN201580.1 (SA17) demonstrates its potential to produce a considerable amount of enzymes capable of degrading polyethylene material in the broth culture. However, for practical application, the fungal strain must also be able to degrade polyethylene material under natural conditions. To test this, the capability of *F. solani* MN201580.1 (SA17) to produce the same amount of enzymes in soil + mulch media was determined. Additionally, the best method for enzyme extraction was tested in solid media. These findings can help pave the way for the practical application of *F. solani* MN201580.1 (SA17) in the biodegradation of polyethylene material.

During the study, the culture replicates were harvested at each incubation period and two different extraction methods were used: buffer extraction and distilled water extraction. The extracted culture

filtrates were then assayed for their respective enzyme activities. The obtained data revealed that the buffer solvent extraction method was the more accurate methodology for enzyme extractions. Filtrates extracted through respective buffer reagents detected a higher amount of activity for each enzyme, indicating that this method is more effective for extracting enzymes from *F. solani* MN201580.1 (SA17) cultures.

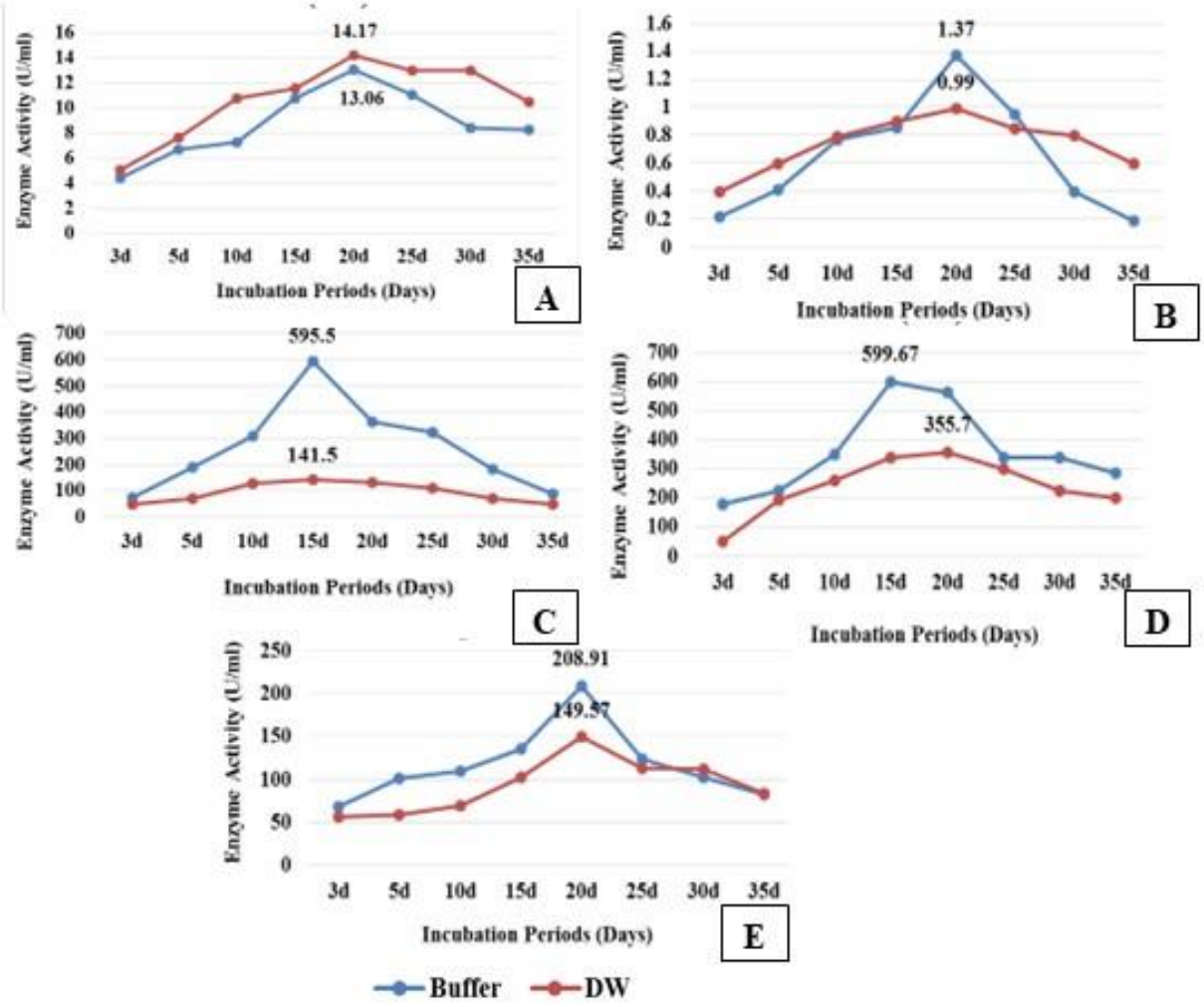

Figure.36. Quantitative analysis of enzyme activities by two different extraction method from solid state medium

A- Laccase activity; **B-** MnP activity; **C-** Protease activity; **D-** Esterase activity; **E-** Lipase activity

The **laccase enzyme** showed higher activity when extracted using distilled water compared to the buffer solvent. On the third day of incubation, the enzyme activity was recorded as 5.07±0.02 U/ml and increased to 14.17±0.02 U/ml on the 20th day. Meanwhile, the enzyme extracted using the buffer method exhibited lower activity, recording 4.42±0.04 U/ml on the third day, with the maximum activity of 13.06±0.04 U/ml observed on the 20th day, as indicated in Table.22 and Figure.36-A. After reaching its peak, the enzyme activity decreased, with 12.97±0.03 U/ml, 12.95±0.08 U/ml, and 10.48±0.03 U/ml noted on the 25th, 30th, and 35th day of incubation, respectively.

Both buffer and distilled water were effective solvents for extracting laccase enzyme. As shown in Figure.36-A, extraction using distilled water yields higher amount (14.17 ± 0.02 U/ml) of laccase enzyme on the 20th day, while 50mM sodium-acetate buffer (pH 5.0) extracted 13.06 ± 0.04 U/ml of activity. Distilled water was more efficient for extracting laccase at each incubation period compared to buffer. In previous studies, Téllez-Téllez et al. (2008) detected 0.8 U/ml and 2.3 U/ml of laccase activity in *P. robustus* 250 strain cultivated in banana peels and kiwi fruits, respectively. *Trametes* sp., *Ganoderma* sp., and *Pleurotus* sp. were also cultivated in different solid media and showed laccase activity of 0.8 U/mL (7 days), 100 U/ml (28 days), and 6.83 U/ml (7 days), respectively (Kaur et al., 2016; Bertrand et al., 2015; Ding et al., 2014). In comparison, *F. solani* MN201580.1 (SA17) produced 6.69 ± 0.02 U/ml of laccase activity in just 5 days of incubation period when cultured in soil and mulch.

Table.22. Evaluation of enzyme activity in Solid State medium by two extraction methodology

Incubation period		**3days**	**5days**	**10days**	**15days**	**20days**	**25days**	**30days**	**35days**
Laccase (U/ml)	**Buffer Extraction**	4.42 ± 0.04	6.69 ± 0.02	7.25 ± 0.03	10.78 ± 0.05	13.06 ± 0.04	11.07 ± 0.02	8.42 ± 0.09	8.23 ± 0.02
	DW Extraction	5.07 ± 0.02	7.61 ± 0.03	10.76 ± 0.05	11.51 ± 0.04	**14.17 ± 0.02**	12.97 ± 0.03	12.95 ± 0.08	10.48 ± 0.03
MnP (U/ml)	**Buffer Extraction**	0.22 ± 0.008	0.41 ± 0.008	0.77 ± 0.01	0.86 ± 0.01	**1.37 ± 0.006**	0.95 ± 0.24	0.40 ± 0.03	0.19 ± 0.07
	DW Extraction	0.40 ± 0.005	0.60 ± 0.005	0.79 ± 0.005	0.90 ± 0.006	0.99 ± 0.01	0.85 ± 0.01	0.8 ± 0.19	0.60 ± 0.01
Protease (U/ml)	**Buffer Extraction**	73 ± 1.15	190.5 ± 1.91	308.5 ± 1.91	**595.5 ± 3**	364.5 ± 1	323.5 ± 3	182.5 ± 1.91	88 ± 1.63
	DW Extraction	48 ± 1.63	70.5 ± 1	128.5 ± 1	141.5 ± 1.91	134 ± 3.16	109 ± 1.15	71 ± 1.15	50.5 ± 1
Esterase (U/ml)	**Buffer Extraction**	178.48 ± 0.73	226.36 ± 0.64	348.34 ± 3.51	**599.67 ± 5.86**	561.6 ± 6.8	339.5 ± 0.55	338.9 ± 0.94	284.4 ± 0.35
	DW Extraction	49.75 ± 0.25	192.32 ± 0.56	260.34 ± 0.54	339.2 ± 0.63	355.7 ± 4.16	298.24 ± 0.66	226.25 ± 0.63	200.2 ± 0.33
Lipase (U/ml)	**Buffer Extraction**	68.91 ± 0.22	101.9 ± 0.19	109.84 ± 0.17	135.22 ± 0.19	**208.91 ± 0.27**	124.03 ± 0.19	102.84 ± 0.23	82.36 ± 0.28
	DW Extraction	56.09 ± 0.21	59 ± 0.13	69.17 ± 0.19	102.94 ± 0.17	149.57 ± 0.26	113.9 ± 0.19	112.62 ± 0.17	83.69 ± 0.19

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

The data presented in Figure.36-B indicates that the buffer reagent extraction method yielded significantly higher levels of **MnP enzyme** compared to the distilled water extraction method. Specifically, on the 3rd day of incubation, the buffer method yielded 0.22±0.008 U/ml of enzyme, while the distilled water method yielded 0.40±0.005 U/ml. On the 10th and 15th day, both methods detected similar levels of enzyme activity, with the buffer method yielding 0.77±0.01 U/ml and 0.86±0.01 U/ml of enzyme, and the distilled water method yielding 0.79±0.005 U/ml and 0.90±0.006 U/ml of enzyme, respectively. On the 20th day, both methods detected peak activity, with the buffer method yielding 1.37±0.006 U/ml and the distilled water method yielding 0.99±0.01 U/ml of enzyme. Previous studies also suggested that solid-state growth media are more suitable for *Fusarium* to yield higher enzymatic activities compared to liquid media (Li et al., 2008; Chang et al., 2012). Manganese peroxidase (MnP) enzyme activity was slightly elevated in the soil+mulch medium compared to the broth culture medium. This fungal strain was able to produce 1.37 ± 0.006 U/ml (Table.22) of MnP after 20 days, which is slightly higher than the activity found in the broth culture. When comparing the two extraction solvents, the 50mM sodium-acetate buffer (pH 5.0) reagent was able to extract a greater amount of enzyme than distilled water. As seen in the figure.36-B and Table.22, the enzyme extracted through the buffer reagent showed 1.37 ± 0.006 U/ml activity, whereas only 0.99 ± 0.01 U/ml activity was detected in the enzyme filtrate collected through distilled water. Iandolo et al. (2011) found 14.55 U/ml and 12.39 U/ml MnP enzyme activity from *Trametes pubescens* and *T. versicolor*, respectively, when grown on banana peels and tree leaves.

The **protease enzyme** exhibited similar levels of enzyme activity in soil and mulch medium as in the broth culture medium when extracted using a 10% ethanol-water mixture (Table.22 and Figure.36-C). The enzyme activity was 73±1.15 U/ml on the third day, and it peaked at 595.5±3 U/ml on the 15th day. The activity decreased to 182.5±1.91 U/ml on the 30th day and 88±1.63 U/ml on the 35th day when extracted using buffer solvent. On the other hand, when extracted using distilled water, the crude enzyme extract showed only 48±1.63 U/ml on the third day, 141.5±1.91 U/ml on the 15th day, and 50.5±1 U/ml on the 35th day of enzyme activity.

In a study by Tunga et al. (1999), the best solvent for leaching protease enzyme from wheat bran solid medium was found to be a 10% ethanol-water mixture, which resulted in 61.5 U/ml of protease produced by *Rhyzopus oryzae* in 6 days. Similarly, García-Gómez et al. (2009) investigated protease activity in *A. oryzae* grown on fish flour and found the fungus produced 0.12 U/ml in two days. In the present study, *F. solani* MN201580.1 (SA17) in soil and mulch growth medium showed remarkable potential in producing large amounts of protease enzyme. As indicated in Table.22, *F. solani* MN201580.1 (SA17) produced 190.5 ± 1.91 U/ml of protease in just five days of incubation, with tactivity peaking on the 15th day and reaching 595.5 ± 3 U/ml in filtrate extracted by a 10% ethanol-

water mixture. In comparison, distilled water was only able to leach out 141.5 ± 1.91 U/ml of enzyme from the soil+mulch. These results suggest that *F. solani* MN201580.1 (SA17) has high potential for producing protease enzyme in soil and mulch growth medium, and that buffer solution is the best leaching solvent for protease assays (Figure.36-C).

The results indicate that Tris-HCl buffer reagent is more effective in detecting **esterase enzyme** activity compared to distilled water extraction method. On the 3rd day of incubation, the buffer method detected significantly higher enzyme activity (178.48±0.73 U/ml) compared to the DW method (49.75±0.25 U/ml). After 15 days, the buffer method detected a peak activity of 599.67±5.86 U/ml, while the DW method detected only 355.7±4.16 U/ml of enzyme activity. The activity declined after the peak, and on the 35th day, the buffer method recorded 284.4±0.35 U/ml of activity, whereas the DW method detected only 200.2±0.33 U/ml of enzyme activity. Overall, these findings suggest that Tris-HCl buffer reagent is more effective than DW extraction in detecting esterase enzyme activity.

The results indicate that *F. solani* MN201580.1 (SA17) has the potential to produce significant amounts of esterase enzyme in medium supplemented with soil and mulch, comparable to that produced in casein broth medium. Extraction of the enzyme with 50 mM Tris-HCl buffer resulted in the highest esterase activity (599.67 ± 5.86 U/ml) on the 15th day, while distilled water extraction yielded a lower amount of enzyme (141.5 ± 1.91 U/ml), as shown in Table.22 and Figure.36-D. In a previous study, *F. oxysporium* was found to produce 7 U/ml esterase in 5 days when grown on a solid medium containing 7% tomato skin powder (Christakopoulos et al., 1998). In comparison, *F. solani* MN201580.1 (SA17) produced a higher esterase activity (226.36 ± 0.64 U/ml) in soil and mulch after 5 days of incubation under optimized conditions.

The **lipase enzyme** produced in solid state growth medium exhibited maximum activity on the 20th day, with 208.91±0.27 U/ml of enzyme detected when extracted using Tris-HCl buffer solution. In contrast, only 149.57±0.26 U/ml of enzyme activity was detected with distilled water extraction on the same incubation period. The enzyme activity levels on the third day were 68.91±0.22 U/ml and 56.09±0.21 U/ml with buffer reagent and distilled water extraction, respectively. However, after its peak, the activity levels of the lipase enzyme reduced to 82.36±0.28 U/ml and 83.69±0.19 U/ml on the 35th day with buffer and distilled water extraction, respectively.

It has been reported in previous studies that *Fusarium* has the potential to produce large amounts of extracellular lipase in solid-state media. Oliveira et al. (2020) found that *Fusarium* grown on crambe cake produced 61.36 U of lipase enzyme activity in just two days, while *Fusarium* grown on solid medium containing 10 gm of almond meal produced 56.7±0.01 U/mL of lipase activity after three days. In the present study, *F. solani* MN201580.1 (SA17) grown in soil and mulch media produced 101.9 ± 0.19 U/ml of lipase enzyme in five days, which is a considerable amount. The lipase enzyme

activity was observed to peak on the 20th day, with 208.91 ± 0.27 U/ml of activity being extracted using buffer extraction and 149.57 ± 0.26 U/ml of enzyme being extracted by distilled water, as shown in Table.22 and Figure.36-E. Tris-HCl buffer solution was found to be more suitable for the extraction of lipase from solid media.

The fungal strain *F. solani* MN201580.1 (SA17) showed an equal amount of enzyme activity in soil and mulch medium indicating the efficacy of this biodegradation process in natural environment. The use of fungal strains, such as *F. solani* MN201580.1 (SA17), which can produce enzymes that effectively break down polyethylene, presents a promising approach to addressing this issue. The development of optimized conditions for enzyme production and polyethylene degradation under natural conditions is a crucial step towards the implementation of effective bioremediation strategies.

Key observations of the study

The optimized conditions for the significant activity of Laccase, MnP, Protease, Lipase & Esterase enzymes are as follows:

Optimum inoculum size: Single disc

Optimum incubation period: 20th day of inoculation

Optimum temperature: 30°C

Optimum pH: pH 8

Medium for evaluation: Soil and mulch medium

Optimum extraction method: Buffer extraction method