CHAPTER 4:

STUDIES ON ENZYMES RELEASED FROM THE SELECTED FUNGAL STRAINS- OPTIMISATION, CHARACTERISATION

4. STUDIES ON ENZYMES RELEASED FROM THE SELECTED FUNGAL STRAINS-OPTIMISATION, CHARACTERISATION

4.1 Introduction

Fungi are known to produce a chemically diverse array of metabolites, many of which are indirectly important for their growth and survival (Toghueo 2020). Conventional methods of biocontrol control of waterhyacinth using fungi have been implemented for decades (Freeman and Charudattan 1984, Ray and Hill 2012, Dutta at al. 2015, Firehun et al. 2017). But the development of alternative weed control measures are required to decrease the resilience of the usage of environmentally damaging chemical herbicide (Singh and Pandey 2019).

Biocontrol of weeds using fungal metabolites have been in limelight for over a long period of time, to date and mostly emphasis has fallen or has been narrowed down to the use of secondary metabolites or mycotoxins (Liu and Li 2004), produced by the host fungi that have been implemented for biocontrol of host weeds. The metabolites are produced by phytopathogenic fungi during or as an outcome of host-pathogen interaction. The use of eco-friendly metabolites have produced an impact on the targeted weed, by elucidating disease manifestation (Vey et al. 2001). This eventually, drew attention on elucidating disease etiology, their characteristics and the mechanism they follow to biologically control the target weed (Brimmer and Boland 2003, Casida 2009, Cimmino et al. 2015).

Other than secondary metabolites fungi that are also known to release primary metabolites, often enzymatic in nature and capable of degrading/damaging plant cell wall, known as cell-wall degrading enzymes (CWDEs) (Kikot et al. 2009, Kubicek et al. 2014). Such fungal enzymes can enable the phytopathogen to penetrate the cell wall by initiating the disintegration of the structural rigidity and backbone of the host plant cell wall (Zeilinger et al. 1999). To overcome the plant cell wall barrier, phytopathogenic fungi produce enzymes, which leads to deconstruction of the structural chemical units like cellulose, xylan, and pectin. The structural moiety consists of hemicelluloses, pectic substances and structural proteins. The middle lamella of cell wall is largely comprised of pectic substances, interconnected with cellulose microfibrils, especially among young plants. It is found mostly with the

backbone of α-1, 4-linked-D-galactonuronic acid residues, forming the homogalacturonan chains (Kikot et al. 2009). While the middle lamella is chiefly of the pectic substances, the primary wall is predominantly constituted of xylan, a major hemicellulosic component, mostly in case of monocotyledonous plants like waterhyacinth. It forms a complex structure with D-xylose backbone linked with β-1, 4-bridges. In addition to cellulose and matrix materials and structural proteins, water fills approximately 50 % of the space in typical primary walls. The secretion of CWDEs play an important role during penetration and disease establishment (Kang and Buchenauer 2000a, b). Phytopathogenic fungi require these enzymes during the later stages of invasion (Gibson et al. 2011). These fungi actively degrade the plant tissue and liberate monosaccharides and oligosaccharides for their growth and reproduction (Kubicek et al. 2014).

Enzyme extraction is an environmentally-friendly process which allows to obtain high yields under optimal conditions (Sabater et al. 2018). Before going into the quantitative measurement of the enzyme and understanding its nature and best suited activity range, it is essential to understand if the fungi is capable of producing the enzyme of interest. Understanding the nature of these enzymes, and enhancing their activity for effective and better functioning can be brought about with optimisation of the enzymatic extraction process using various desirable function and parameter changes. The highest concentrations of respective sugar content corresponds to the high functionality, activity and purity of the enzyme.

The chapter highlights at the enzymatic production of phytopathogenic fungi and utilisation of these optimised enzymes to evaluate their biocontrol potential and respective functionalities.

4.2. Materials and methods

4.2.1. *Potential strains*

Two potent phytopathogens showing high biocontrol potential against the invasive weed, waterhyacinth (observed previously in **Table 3.3**, **Chapter 3**), among the plethora of phytopathogens isolated, based on their disease intensity and disease severity indices, were characterized based on their morphology, growth and cultural characteristics (in both macroscopic and microscopic views of the culture) (methodologies of which have been highlighted in **Chapter 1**). The disease symptoms caused by these phytopathogens infecting waterhyacinth were also recorded based on their visual characteristics.

The stock cultures of the isolate were stored at 3 ^{o}C on PDA slants. Fresh PDA slants were produced every month and used in these studies.

4.2.2. *Fermentation conditions*

The slants having the stock culture of the phytopathogens were thawed and then transferred on sterile PDA plates (preparation of which is mentioned in **Annexure I-1.2**). For *in vitro* analysis, the fungal cultures were grown in Potato Dextrose Agar media plates. Each Petri plate was inoculated with two disks (8 mm diameter) of each fungus species 45 mm apart, obtained from the growing margins of pure colonies of *A. alternata* and *F. oxysporum* sp. *lycopersici* 4287 grown in PDA at around 27 ^{o}C for 5-7 days, maintaining a humidity of 70-80 %; plates were incubated for 2-3 weeks in similar conditions as stated earlier. Petri plates were examined time to time, for avoiding and eradicating contaminants.

4.2.3. *Enzyme assays*

4.2.3.1. *Qualitative analysis*

For qualitative assays, uniform inoculation procedure is necessary. To obtain inoculum the test fungi were cultivated on basal medium (**Annexure I-1.2**) supplemented with 0.4 % w/v glucose and solidified with 1.6 % w/v agar. A single agar

disc cut from the actively growing colony margin of a culture to inoculate each assay medium.

For Cellulolytic enzyme assays,

Cellulolysis basal medium (CBM) (g/l in distilled water)

$C_4H_{12}N_2O_6$	5	Yeast Extract	0.1
KH_2PO_4	1	$CaCl_2.2H_2O$	0.001
$MgSO_4 \cdot 7H_2O$	0.5		

Basal medium is stored as 10x sterilized stock. The basal medium can be substituted with peptone plus yeast or malt extract with a concentration of 0.1-0.2 % w/v to support cellulose degrading activity of the fungi.

Among several methods of cellulolytic enzyme production detection, dye staining of carboxymethylcellulose (CMC) agar method (Pointing 1999) was used. The protocol is as follows:

1. The CBM medium is supplemented with 2 % w/v CMC (of low viscosity) and 1.6 % w/v of agar and autoclaved
2. The medium is aseptically transferred to sterile Petri plates
3. The plates were the inoculated with the test fungi
4. The plates were then incubated at 25 ^oC in darkness. When the colony diameter is around 30 mm, which usually takes 2-5 days, the plates were stained as follows,
5. The plates were flooded with 2 % w/v aqueous Congo red (C.I. 22120) and left for 15 minutes.
6. The stain was then poured off and the agar surface washed with distill water
7. They were then flooded with 1 (M) NaCl for 15 minutes, for destaining.
8. After which the counter-stain was then poured off.

For Hemicellulolytic (xylanolytic) enzyme assays

Xylanolysis basal medium (XBM) (g/l in distilled water)

$C4H_{12}N_2O_6$	5	Yeast Extract	0.1
KH_2PO_4	1	$CaCl_2.2H_2O$	0.001
$MgSO_4.7H_2O$	0.5		

Basal medium can be stored similarly as stated in the CBM storing method.

Dye staining of xylan agar (Pointing 1999) method was used and the staining procedure is as follows:

1. The XBM medium is prepared by adding 4 % w/v of xylan and 1.6 % w/v of agar and autoclaved.
2. The medium is aseptically transferred to sterile Petri plates, which is then cooled until it becomes viscous and gently mixed before pouring, to ensure uniform distribution of xylan in the agar medium.
3. The settled plate is then inoculated with the fungi to be tested
4. Then the plates are incubated in 25 ^oC darkness until the colony is around 30 mm (2-5 days old).
5. For staining, the plates were then flooded with iodine stain (0.25 % w/v aqueous I_2 and KI) and then left for 5 minutes.
6. The stains are poured off and the agar surfaces are washed with distilled water

   To help understand if the phytopathogen produces a significant amount of enzyme, to be carried to the next phase of the experiment for quantitative assay, a simple scoring system with few categories (e.g., no reaction-weak reaction-strong reaction) was used.

4.2.3.2. *Quantitative assay*

The potential strain/s cultivated in Potato Dextrose Broth (PDB) were inoculated in 100 ml Erlenmeyer flasks each containing 10 ml Basal Medium (BM) composed of (g/l) (as follows)

Peptone	0.9;	$(NH_4)_2HPO_4$	0.4;
KCl	0.1;	$MgSO_4.7H2O$	0.1;
$K_2Cr_2O_7$	trace;	carbon source	10

Depending on the type of enzyme to be extracted, varying carbon sources like pure carboxymethyl cellulose, cellobiose, xylan or pectin were used for β-glucosidase (EC 3.2.1.21), endoglucanase (EC 3.2.1.4), xylanase (EC 3.2.1.8) and polygalactonurase (EC 3.2.1.15) respectively at pH 7 and incubated for 48 hours at 27 ^{o}C, in BOD.

The fermented culture media, bearing the enzyme, were then centrifuged at 10,000 rpm for 10 min and the supernatant used as the crude enzyme. To measure the activity of each type of enzyme, the assay mixture (1 ml) containing an equal volume of enzyme and 1 % w/v of respective substrate dissolved in 0.1 (M) phosphate buffer (pH 7) (**Annexure I-1.1**) was incubated at 27 °C for 10 min. For assaying the activity of β–glucosidase, endoglucanase, endoxylanase, polygalactonurase (PGase) the respective substrates (as mentioned earlier) were used.

To understand the activity of the enzyme, the assay mixture (1 ml) containing an equal volume of enzyme and 1 % w/v of respective substrate dissolved in 0.1 (M) phosphate buffer (pH 7) incubated at 37 °C for 10 min, was measured. The reducing sugar released in each case, was measured by the dinitrosalicylic acid (DNSA) method (**Annexure I-1.3**) (Bernfeld, 1955), and the absorbance of the enzymes was correlated using a standard (glucose) product curve to quantify the concentration (in Enzyme Unit/ml or U/ml) (**Annexure I-1.3**). In case of PGase activity one unit of enzymatic activity (U) was defined as one μmol of galactunoric acid released per minute (Trejo-Aguilar et al. 1996).

Blanks were prepared with inactivated enzymes. One unit of the enzyme activity is referred to the amount of enzyme producing one micromole of product per minute

under the assay conditions. β–glucosidase, endoglucanase, endoxylanase, activity of the culture supernatant was determined as described by Ray et al. 2013. PGase activity in the cultural filtrate was determined according to the method of Sur et al. (2014).

4.2.4. *Biocontrol potential of the fungal enzymes*

A part of the crude enzyme was used to check the activity of each type of enzyme and part to check the biocontrol potential and pathogenicity of the enzymes produced, against waterhyacinth. The disease intensity was measured, based on a pictorial disease scale (Freeman and Charudattan, 1984), as mentioned previously in **Chapter 3**.

Statistical analysis

The enzyme activity data was replicated subjected to mean and standard error plotted on the graph. The data, presented with mean and SEM (Standard Error of the mean), recorded in form of percentage, evaluated the damage of different biocontrol agents on the weed.

4.2.5. *Optimisation and characterisation of the enzymes*

The fungi, which produced potent enzymes showing better pathogenicity and enzyme activity were selected for optimisation and characterisation. The concentrations of different substrates used as sole carbon source, was varied from 0.5 % - 3.0 % to optimise the substrate concentration of submerged culture of the potent fungi. The optimum pH for enzyme production was determined by adjusting the initial pH of the fermentation media at a range from 4.0-7.0 using citrate, phosphate and Tris glycine buffers (**Annexure I-1.1**). Most favourable production temperature was studied by incubating the cultivation medium at varying temperatures of 7 °C, 17 °C, 27 °C, 37 °C and 55 °C. The time course of growth and enzyme production by the strain under optimised culture conditions was studied by checking the enzyme production kinetics for 0 to 144 hours at 27 °C.

The crude enzymes in their optimised condition were further analysed and characterised based on their disease-causing potential against fresh untreated host weed, waterhyacinth. The experiment was undertaken via measuring the disease

intensity, based on a pictorial disease scale (Freeman and Charudattan, 1984), as mentioned previously in **Chapter 3**.

4.3. Results

4.3.1. *Potential strains*

The potent two fungal pathogens holding the best results for biocontrol potential were *Alternaria alternata* (WHK-3) and *Fusarium oxysporum* sp. *lycopersici* 4287 (WHK-59) (Stated earlier in **Chapter 3**).

4.3.1.1. ***Alternaria alternata* (WHK-3)**

Alternaria alternata was observed to infect the waterhyacinth lamina and parts of the petiole with large and deep necrotic spots. They were small, circular and irregular spots of about 2-4 mm size. They formed light brown patches, with their concentric zonation. With the more intrinsic the circle, the more would be the impact of necrosis and severity from it. Zones of chlorosis are, at times, observed around the spots of necrosis, on the infected leaves.

Morphologically in Petri plates, the fungus have profuse mycelial growth, which is initially hyaline and then turn greyish brown (**Figure 4.1B**). The plates are often found to be filled with red-metabolites, which are secreted by the fungus itself, turning the entire growing medium reddish in colour (**Figure 4.1A**). Under the microscope, the spores are multi-celled, septate and irregularly branched. The hyphae are thin of about 2.8 µm in diameter, which becomes slightly thick of around 4.48 µm (in diameter) as they age. The length varies from 78.44 to 28.56 µm depending on the age of the spore. Condiophores (**Figure 4.1 C**) appear singly or in clusters. They were light olivaceous to dark brown in colour, varying in shape from obclavate to mostly ellipsoidal, muriform having tapered apex with 1- 3 longitudinal and 2-10 transverse septa.

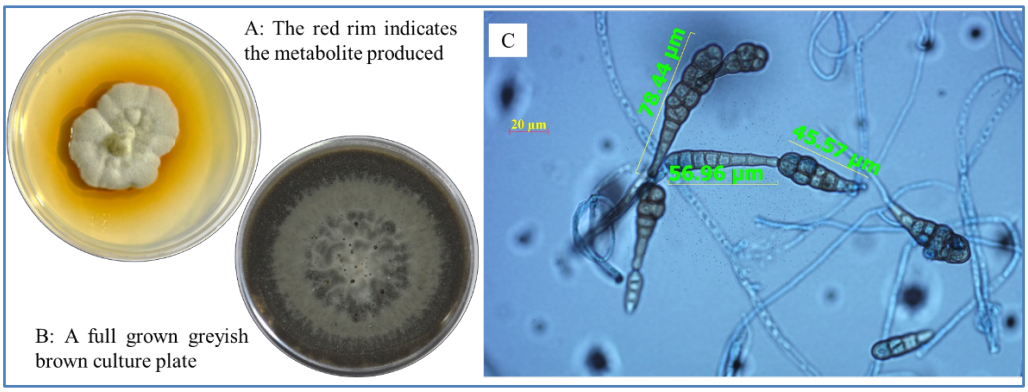


Figure 4.1: **A:** Fungal plate of *Alternaria alternata* (WHK-3) showing an outer rim of production of metabolite; **B**: A fully grown plate of the pathogen; **C:** Microscopic view of the phytopathogen under 40X magnification under compound microscope (with scale bars)

4.3.1.2. ***Fusarium oxysporum* sp. *lycopersici* 4287 (WHK-59)**

Fusarium oxysporum sp. *lycopersici* 4287 infections on waterhyacinth plant appear as deep purplish to black spots, usually seen as severe necrotic spots or flecks on the lamina or/and petiole of the plant. The patches are seen usually to develop around the laminal end of the host, and then spreading across, until the entire leaf is eroded and disintegrated.

Colony characters of the isolate showed purple and white colonies on the PDA plates, fluffy growth, which with age slowly became more suppressed to the media it was growing in (**Figure 4.2A**). Alternating rings of colony colour and aerial growth were observed. Under the microscope crescent shaped (fusoid-subulate and tapered on both ends), septate, hyaline spores were observed at high magnification (**Figure 4.2 B** and **C**). Microspores (or microconidia) and macrospores (or microconidia) were observed simultaneously and often in clusters. Macroconidia are thin-walled bearing three-five septa. The macroconidia measured 15-35 µm in length and around 2.5-4 µm in width, while the microconidia were borne on simple phialides arising laterally, oval to ellipsoidal, straight to curved ranging from 2.5-12 µm in length and around 2-2.5 µm in breadth. Septate are usually missing for the microconidia.

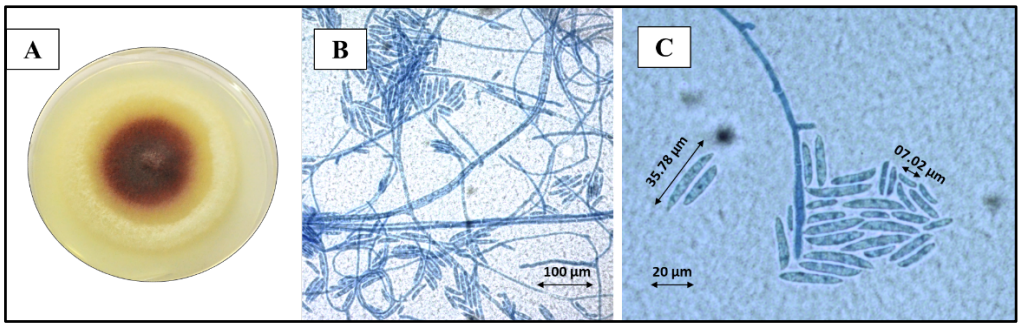


Figure 4.2: A: The fungal plate of *Fusarium oxysporum* sp. *lycopersici* 4287 (WHK-59); Microscopic view of the phytopathogen under **B:** 10X and **C**: 40X magnification under compound microscope (with scale bars)

4.3.2. *Enzyme assays*

4.3.2.1. **Qualitative analysis**

CMC degradation around the colonies appeared as a yellow-opaque area against a red colour for undegraded CMC, around the zone of the colony of the culture (**Figure 4.3**) for both AA and FO, indicating the production of cellulase group of enzymes. The yellow opaque area slowly disbands, but the prominence is more visible in AA to that of FO.

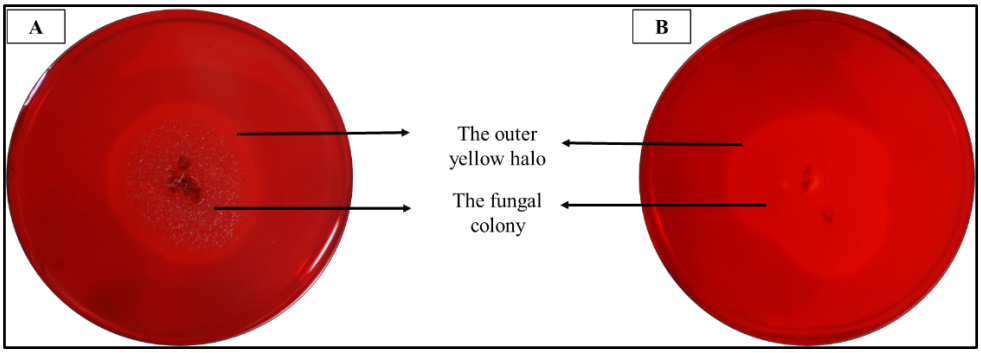


Figure 4.3: The region outside the yellow halo demarcating the un-degraded CMC (A= AA and B= FO)

Appearance of yellow-opaque area against a blue / reddish purple colour for undegraded xylan, hinted at the xylan degradation (**Figure 4.4**) around the phytopathogenic colonies of both AA and FO, inferring that xylanase group of enzymes like endoxylanase and β-xylosidase were produced by both the fungi. The halo or rim outside the colony were prominent and distinctive for FO.

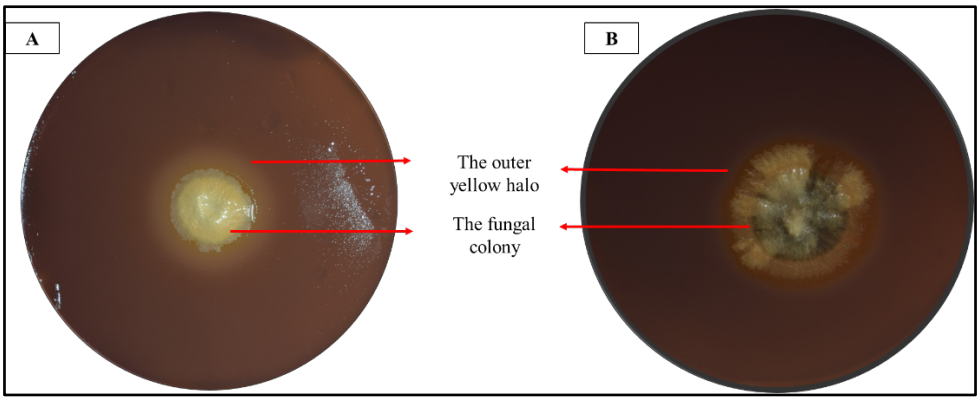


Figure 4.4: The blue / reddish purple region outside the yellow halo demarcating the un-degraded xylan (A= AA and B= FO).

The positive indication of appearance of zone of clearance around the culture, after staining irrespectively, hinted at the production of cellulase and hemicellulase group of enzymes and hence were further quantified for the next step of experiment.

4.3.2.2. Quantitative analysis (along with their biocontrol potential)

For AA, PGase has high activity rate of 1369.69 U/ml, followed by endoxylanase with 646.17 U/ml activity. β–glucosidase had activity a little less to that of endoxylanase of 400.00 U/ml. Endoglucanase (90.87 U/ml) and cellulase (36.63 U/ml) showed very little activity being less than 100 U/ml **(Figure 4.5**).

For the biocontrol potential of the enzymes extracted from AA, β–glucosidase (70.6 % ± 11.85) followed by endoxylanase (64.8 % ± 9.43) and polygalactonurase or PGase (50 % ± 10.65), have shown effective pathogenicity against waterhyacinth. However, the quantity of β–glucosidase is much less than that of endoxylanase and PGase (**Figure 4.5**).

In case of FO, PGase had the highest active of 814.59 U/ml, followed by β–glucosidase with 320.00 U/ml activity. Endoxylanase or xylanase had an activity of around 112.44 U/ml, while endoglucanase had activity (40.00 U/ml) much below 100 U/ml (**Figure 4.6**), as seen in case of AA too.

The pathogenicity trials of the enzymes from FO showed that endoxylanase (81.8 % ± 8.65) showed maximum biocontrol potential, followed by PGase (58.0 % ± 6.44) and β–glucosidase (57.6 % ± 10.16), as evident from the disease index (**Figure 4.6**)

against the host weed. However, endoxylanase have a much lesser activity in terms of PGase, its pathogenicity rate and the disease incurred by the enzyme was much higher.

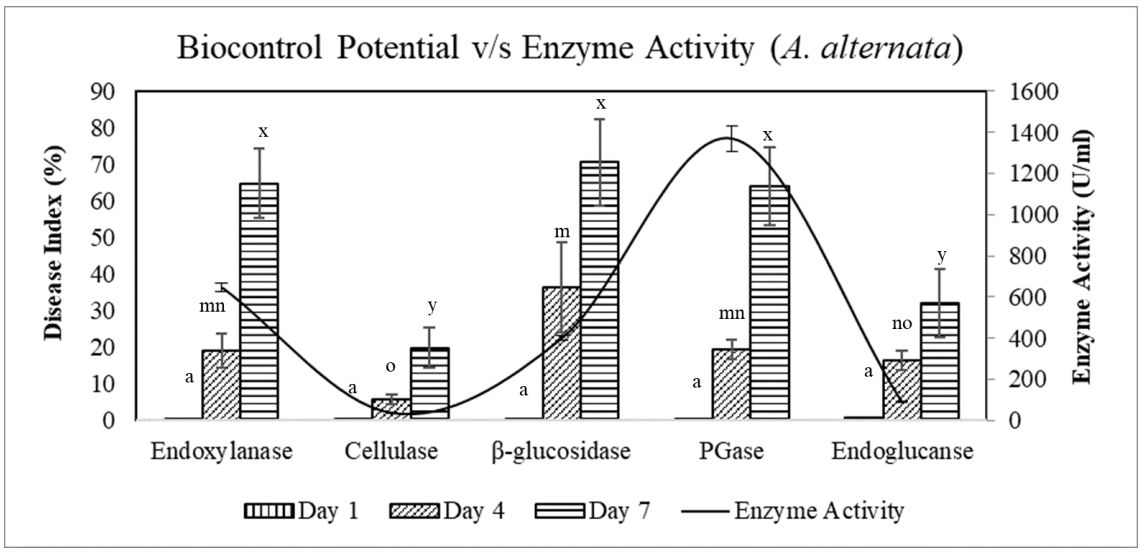


Figure 4.5: Pathogenicity and activities of various enzymes produced by *A. alternata* (Mean values having different superscripts are significantly different (P<0.05))

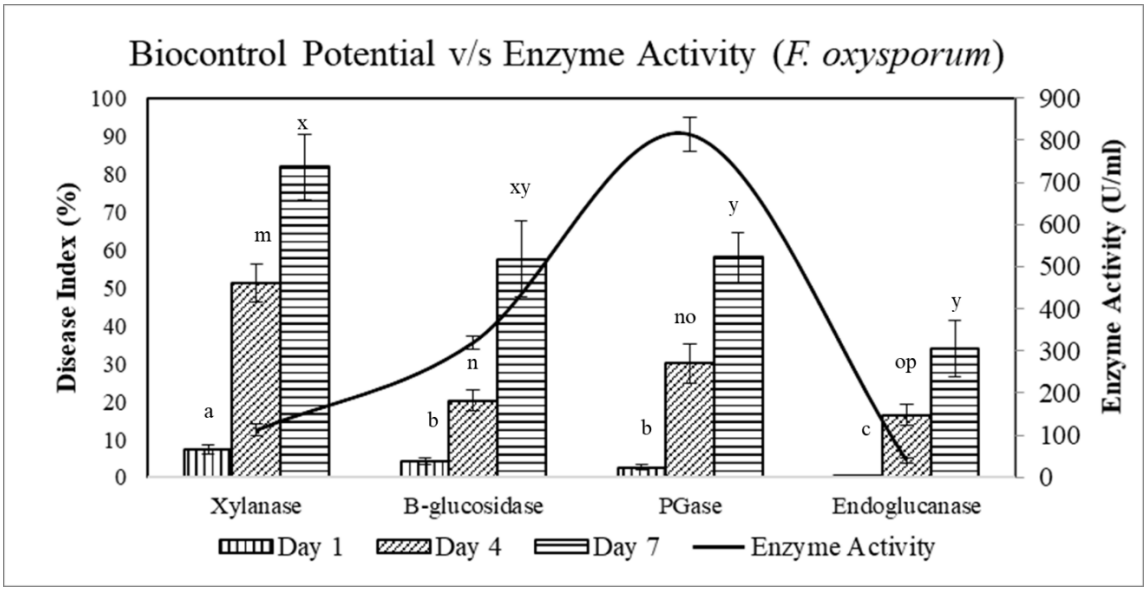


Figure 4.6: Pathogenicity and activities of various enzymes produced by *F. oxysporum* sp. *lycopersici* 4287 (Mean values having different superscripts are significantly different (P<0.05))

4.3.3. *Optimisation of the fungal enzymes*

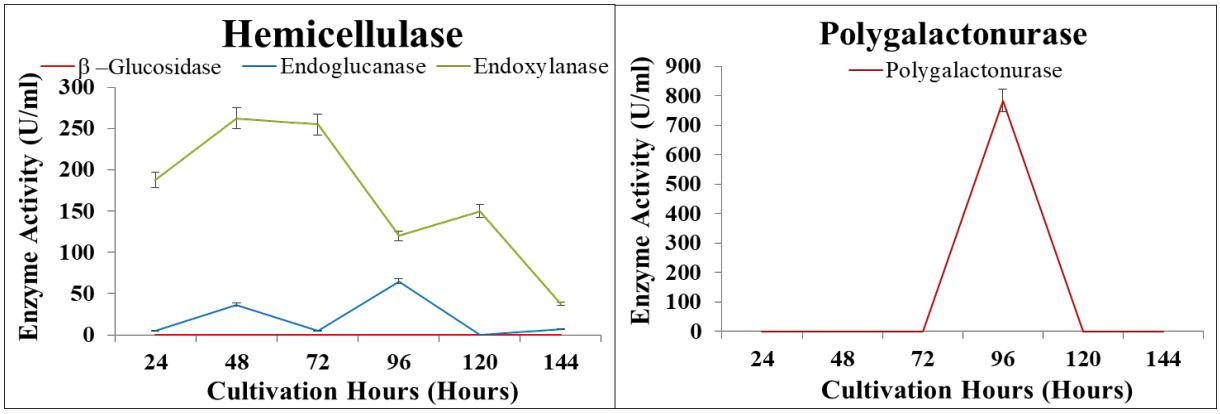
Enzymes of FO and AA have both shown potential against the target weed, but due to high level of autotoxicity of the latter, maintenance of the fungal culture was difficult and hence only enzymes from FO were used to watch their potentiality and tissue level effect on waterhyacinth. Apart from that, narrow host-range effect and minimalistic effect on other biocontrol agents have led to the selection of FO.

On optimizing the cultivation or incubation time, for the hemicellulase group of enzymes released from FO (**Figure 4.7 A**), the most favourable time for endoxylanase (EC 3.2.1.8) (with an activity of 262.37 U/ml) and endoglucanase (EC 3.2.1.4) (with an activity of 65.00 U/ml) was 72 hours and 96 hours respectively. Fungus failed to produce β–glucosidase (EC 3.2.1.21). Polygalactonurase (EC 3.2.1.15) produced, was highly active after 96 hours with an activity of 783.26 U/ml.

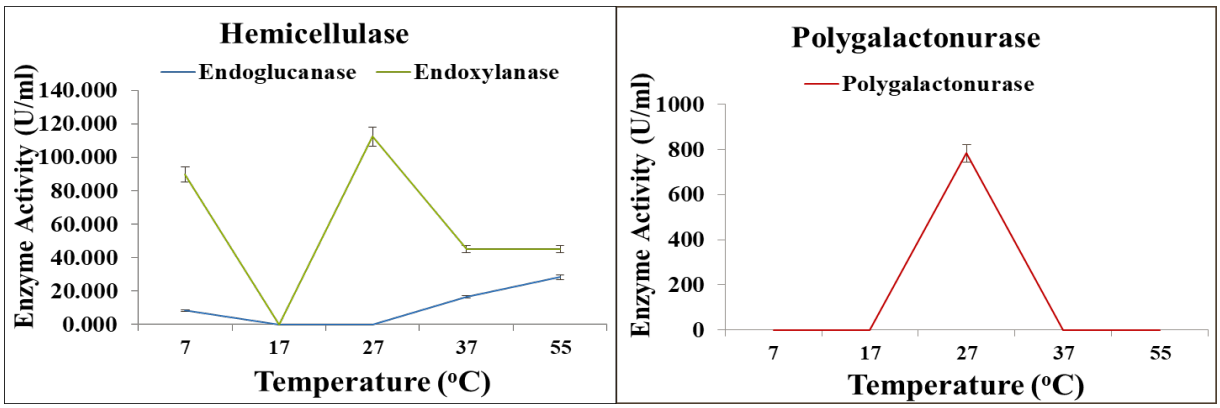
Endoxylanase, from FO, were active at low temperature of 7 ^{o}C (with 6.01 U/ml enzyme activity) and even at 27 ^{o}C (with 112.44 U/ml enzyme activity). When the activity of endoxylanase ceased after 27 ^{o}C, endoglucanase slowly rose with increase of temperature after 27 ^{o}C, reaching to 28.33 U/ml activity at around 55 ^{o}C. PGase, with an activity of 783.26 U/ml was highly active at 27 ^{o}C (**Figure 4.7 B**).

On varying the concentrations of different substrates, endoxylanase (with 71.51 U/ml enzyme activity) and polygalactonurase (with 3263.56 U/ml enzyme activity), both showed its optimum activity in growth media containing 1.5 % of pure xylan and pectin, while endoglucanase using 0.5 % (w/v) of carboxy methyl cellulose (CMC), showing an activity of 16.67 U/ml (**Figure 4.7 C**).

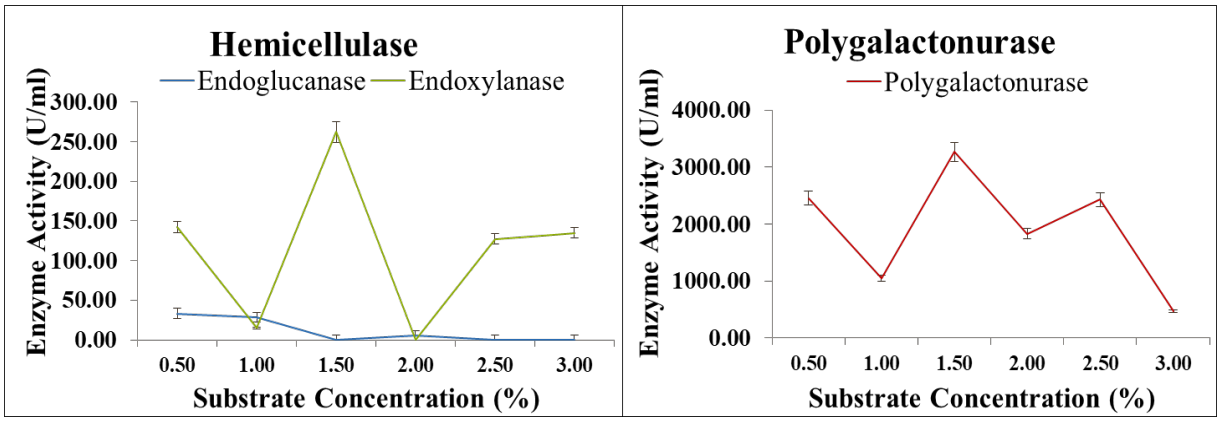
All the active enzymes produced showed their optimised working activity at a lower pH, favouring acidic growing condition. Endoxylanase showed it best activity with 119.94 U/ml at an acidic pH of 4. Endoglucanse showed highest activity at highly acidic pH showing the optimum activity of 25.00 U/ml, which drops with the decrease in acidic level, till pH 6 from where a little surge in activity is observed from neutral pH which declines eventually. PGase showed a sharp peak of activity at a pH medium of 4 with an activity of 2767.50 U/ml (**Figure 4.7 D**).



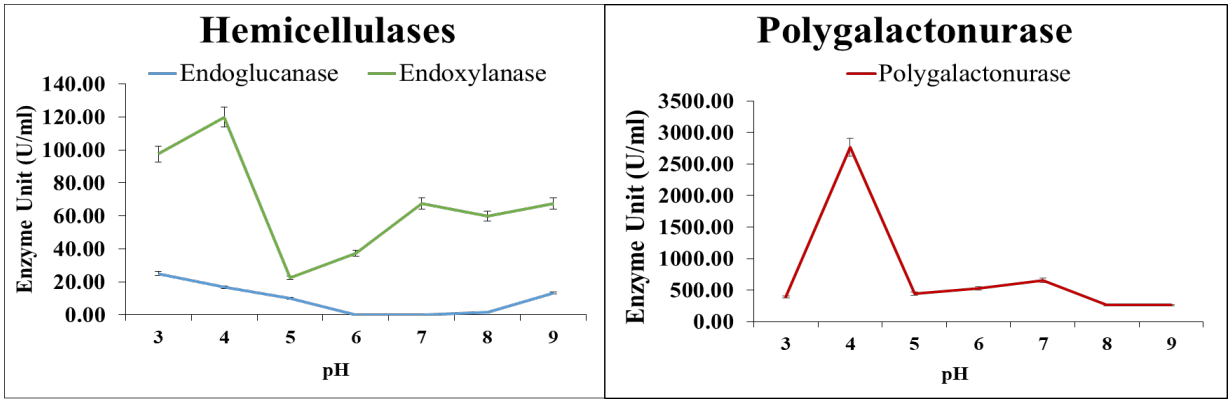
A. Optimisation of incubation time



B. Optimisation of Incubation temperature



C. Optimisation of substrate concentration



D. Optimisation of pH of the growing medium

Figure 4.7: Optimisation of the fungal enzymes on varying growth conditions

4.3.4. *Characterisation of the fungal enzymes (by pathogenicity tests of the enzymes in their optimised condition)*

Partially purified optimized endoxylanase have shown the maximum disease-causing potential (**Figure 4.8**) with 70.6 % damage within 7 days of application of the enzyme, with early onset of infection. It was followed by polygalactonurase which though initiated at a similar pace (with 0.32 % of damage) as endoxylanase but showed an impact on the weed by leaving a 50.00 % damage intensity after the 7 days of application. β–glucosidase and endoglucanase, from FO, followed the impact intensity with 45.78 % and 32.00 % respectively. Endoxylanase have shown effective results against the weed, waterhyacinth, causing infections within a week of application (**Figure 4.9**).

Figure 4.8: Disease symptoms caused by endoxylanase on fresh waterhyacinth plants

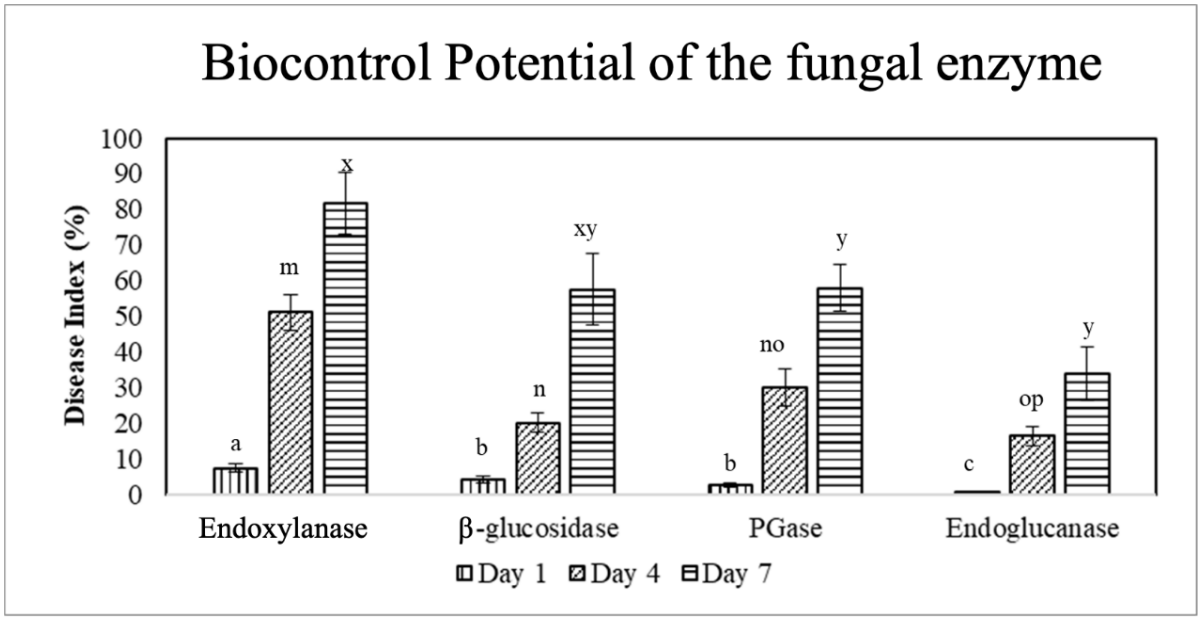


Figure 4.9: Pathogenicity test of the fungal enzymes (Mean values having different superscripts are significantly different (P<0.05))

4.4. Discussion

Among the plethora of fungal secondary metabolites produced and used for biocontrol of the weed, waterhyacinth, certain fungal pathogens enzymatically detoxify the saponins present in the secondary metabolite produced by plants, because they form complexes with the membrane sterols, leading to disintegration of the membrane of plant, and on such interaction, infection susceptibility the host range specificity of the fungi is dependent (Bowyer et al. 1995). To break the structural moiety of the plant cell wall, CWDEs are secreted across the fungal plasma membrane (Kubicek et al. 2014). Pathogens, like *Alternaria* sp. and *Fusarium* sp., which do not have penetration structures, like haustoria, are more likely to secrete enzymes for the invasion and infecting the host plant (Gibson et al. 2011). The selected pathogens also showed very host-specific and effective disease-causing potential, which led to choosing them for extraction of their metabolites (CWDEs).

Qualitative analysis of the CWDEs, produced as a result of degrading the structural polysaccharides, highlights on the component of the host cell wall. Carboxy-methyl-cellulose (CMC) is the respective substrate for endoglucanase and hence their presence in the cell wall is demarcated by the presence of endoglucanase and β-glucosidase. The cellulolytic enzyme assay is a good indicator of cellulolytic ability since endoglucanase

is generally produced in larger titres by fungi than cellobiohydrolase (Cai et al. 1994, Buswell et al. 1996, Pointing et al. 1999a). As seen in case of qualitative analysis for both the pathogens, after growing them on CMC, a dye is used to differentiate between intact non-degraded CMC (stained in purple) and clear degraded substrate. Zinc chloride are also used in place of Congo red dye in many cases (Sass 1958, Gessner 1980). For xylanolytic enzyme assays, the zone of clearance around the culture highlights the utilization of xylan indicating the degradation of the substrate by endoxylanase and β-xylosidase. The efficacy of this staining procedure is limited to the use of birchwood xylan (Egger 1986). Commercially available oat spelt xylan is anticipated to provide good results.

The plant cell wall complexity is reflected by a diverse array of CWDEs produced by fungi (Glass et al. 2013). Where qualitative assay demonstrates the presence of particular CWDEs, the quantitative analysis on the other hand demarcates the difference in the amount of the enzymes produced and relatively the quantification of their respective substrates in the cellular wall component of the host plants (Kubicek et al. 2014), here the weed waterhyacinth, which are degraded by their respective CWDEs.

Host specific metabolites are unique and are effective only to plants that are susceptible to the fungi that produce those (Babu et al. 2003). No significant correlation was drawn where the pathogenicity of the enzymes could be attributed to the enzyme activity (Schäfer 1994). Metabolites from and *Alternaria* itself have been reported earlier too for their autotoxic nature (Saxena and Pandey, 2009) and for that reason, and the survival of the fungi became so hectic that enzymes of *F. oxysporum* was chosen only for working to understand their biocontrol potential.

The fungal enzymes' activity from *F. oxysporum*, when grown on various media showed their best activity in Rose Bengal Chloramphenicol. The influence of culture media may be a significant factor in the production of a highly effective inoculum needed for control of waterhyacinth (Babu et al. 2003). Polygalactonurase of *F. oxysporum* showed optimum activity for temperature at 27 ^{o}C like *Aspergillus* sp. (Galiotou-Panayotou et al. 1997). Polygalactonurase showed maximum production within 96 hours after which exhaustion of nutrients in the medium resulted in drop of activity. Endoglucanase and endoxylanase activity is usually, among many fungi, after

80 hours and 48 hours of inoculation (Ray et al. 2013). When endoxylanase works best at lower temperature, β–glucosidase can work at higher temperature, though the later was not produced by *F. oxysporum* (Ray et al. 2013). All enzyme produced are usually restricted using lower substrate concentration, cause increase in substrate concentration result in drastic reduction in enzyme production probably due to catabolic repression of biosynthesis (Omojasola et al. 2008) or as a result of reduced mass transfer of oxygen by higher amount of solid substrate (Ghosh and Ray 2011). The amount of mycelium present was directly responsible for the quantity of uptake of cations and anions and efflux of ammonia (Xue et al. 2018), which affected the pH, hence a lower pH with large number of ions is preferable for the enzymes to work efficiently and actively.

The most favourable production temperature, optimum pH, and time course of growth and enzyme production by the selected strain under optimized culture conditions for enzyme production was determined, in order to understand the effective active conditions of these primary metabolites.

The presence of CWDEs has been reported in healthy tissue in several plants (Jayasinghe et al. 2004, Chaurasia et al. 2014). Similar to the trend, traces of CWDE activity were detected in healthy tissues in our study (as the control sets of treatment did show pathogenicity effects when applied on waterhyacinth), but we cannot exclude that this trace activity was due to the experimental conditions, for example, the use of a clear plastic bag to maintain high humidity for plant growth. Although plants activate the immune system when they encounter pathogenic microorganisms, including secreting enzyme (Shafikova and Omelichkina 2015) for the current study, the plant tissues were necrotic due to infection by the pathogen. Therefore, we suggest that it is very likely that the major contribution to the overall quantity of the CWDEs present in the diseased tissue extracts is of fungal origin.

4.5 Conclusion

Enzymatic degradation of plant polysaccharides have been used in fuel and many other industries but no such literature of CWDEs associated with weed biocontrol, has been reported. Hence aiming to focus on the potentiality of such metabolites against this weed, the possibilities of studying the enzymes and enhancing their activity

through physiochemical studies to improve their bioherbicidal potential against waterhyacinth, needs to be studied further to understand the mechanistic approach of the working of these enzymes. Plant-fungi interaction and the functions of each individual enzyme (CWDE) requires to be studied independently with detailed information about the composition and characteristic of plant cell wall, regulation of gene expression and enzyme activity of the CWDE, and the interaction needs to be further analysed between the plant cell wall and CWDE.