CHAPTER 6:

SUMMARY OF WORK, CONCLUSION AND FUTURE PERSPECTIVES

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6.1. Summary of work

Waterhyacinth is one of the top ten most invaded and dreaded aquatic weed around the globe and its rising infestation rate and engulfing of water bodies and making them unfit for any kind of use have drawn into means for controlling their extensive growth, which occurs at the cost of disrupting the ecological and environmental harmony. The beautiful flower of this '*Terror of Bengal*' have lured many as a reason for ornamentation and have entered various mainland and have conquered the waterbodies, causing menace. It has anthropogenically spread to various tropical and subtropical parts of the world resulting in it becoming an invasive weed causing loss of 100 million US Dollar annually. The pros of the weed on being used as food or in various cottage industries have been overshadowed by many negative aspects of the weed's infestation that have left a higher impact at the ecological, economical and hygienic forefront. In order to regulate the uncontrollable spread of this weed, several measures have been implemented- initially by manual and mechanical measures, followed by the use of several weedicides. Manual methods have failed to cope up with their fast growth rate while chemical control methods cause negative effects on the non-target species. This has led to the incorporation of biocontrol agents. Biocontrol agents are eco-friendly and effective but are often slow and unpredictable. Hence an intensive search for new agents is needed, with special importance to indigenous ones, that can cumulatively bring down the infestation rate of this weed. During this study, extensive search, purification and identification of a plethora of microorganisms was done. Many newly potent phytopathogens were added to the list of biocontrol agents, which further aggravates the overall control of the weed.

Several phytopathogens were prominently visible on isolation from infected leaves of waterhyacinth and attempts were made to categorise their role as primary and secondary infection-causing agents. The sole identification of potent biocontrol agent could only be tested by pathogenicity and the efficacy trials against the weed itself. On the basis of disease intensity and disease severity index four fungi including *Alternaria alternata* (AA/WHK-3), *Fusarium oxysporum* sp. *lycopersici* 4287 (FO/WHK-59), *Fusarium verticilloides* (FR/WHK-74) and *Paradendryphiella salina* (PS/WHK-37), were found to the most effective among the array of phytopathogens isolated and tested against the macrophyte. Apart from the disease severity, in order to understand the impact of the agents on the surrounding environment, the performance of host-range (Dutta et al. 2015) and interaction among the agents themselves (Dutta and Ray 2017) are essential before using them for field trials. The phytopathogens have a very narrow host-range, affecting mostly other aquatic weeds. AA, PS and FO impacted *Spinacia* sp. and *Chenopodium* sp., among cultivated weeds (so while field trials, one must be mindful of their presence in the surrounding). In the case of FR, it is mostly restricted to other weeds. Studying interaction among biocontrol agents, it was seen that within 24 hours of application, FO along with other agents (NB-*Neochetina bruchi* and OT-*Orthogalumna terebrantis*), started to show their effect on the weed applied in comparison to other agents applied singly or in combinations. By 72 hours, the trend of action in different combinations were prominent. PS+NB and FR+NB showed a decline while their respective phytopathogens showed a higher impact against the weed. In fact the presence of PS also caused also caused the death of the weevils, NB. And the same pattern was carried till the end of the experiment after 120 hours. The result of this study was a reason for opting out or not favouring FR and PS, from the next phase of the studies.

Different control measures, in the form of manual and chemical methods and then biocontrol (first arthropods, followed by fungi and their metabolites) have been implemented to control this weed. Since primary metabolites are responsible for cell wall degradation they can be useful in causing initial damage to the target weed, exposing the plant tissue prone to secondary infections. The primary metabolites of AA and FO were initially observed qualitatively and FO showed best results, in terms of biocontrol potential, while AA was opted out further for their autotoxic nature. Primary metabolites of FO were then subjected to optimisation in order to understand the favourable conditions at which they are highly active and capable of giving peak performances. On varying the cultivation time, endoxylanase showed best activity at the cultivation period of 48 to 72 hours, endoglucanase after 96 hours, while β–glucosidase showed no activity at all, among the hemicellulase group of enzymes. Polygalactonurase on the other hand gave best activity at 96 hours. In case of varying the incubation temperature, the most favourable temperature for hemicellulase (endoxylanase and endoglucanase) activity was found to be at a lower temperature like 7 °C and rising again at 27 °C. Polygalactonurase showed its best activity at 27 °C.

Optimising substrate concentration, it was found that endoglucanase and endoxylanase showed their optimum activity at 0.5 % and 1.5 % (w/v) using pure CMC and xylan respectively. Polygalactonurase showed maximum activity using 1.5 % (w/v) pectin as source. Lastly on optimising pH of the growing medium, it was found that endoxylanase and endoglucanase showed their optimum activity at highly acidic and well as near neutral pH condition. Polygalactonurase showed the best activity at an acidic medium of around pH 2. Further on using the optimised enzymes of FO to check the pathogenicity against waterhyacinth it was found endoxylanase, followed by polygalactonurase showed good results against the weed. Endoxylanase, hence with the best results, was used for the next phase of the experiments.

Understanding their mode of action and evolutionary significance becomes equally important to understand the functional perspective of any biocontrol agents, especially metabolites and so, the biocontrol potential of the enzyme was observed at the tissue level. Dissolution and disintegration of the cell membranes was observed after being incubated with partially purified endoxylanase and also slow detachment of the epidermal layer of the waterhyacinth leaf was evident in the transverse section of the endoxylanase treated leaf. On aiming to investigate for the genes responsible for such cell-degradation behavior, we could see prominent thick bands in xylan treated samples compared to thinner bands at non-treated samples, indicating higher expression, by the activation of the particular enzyme producing genes (XYL2, XYL3 and XYL5). With the passage of time, various organisms adapt and evolve, in order to survive and exist and that has been significantly observed in the evolutionary lineage. Hence, in order to understand the evolutionary significance of the responsive xylanase genes, phylogenetic analysis was done. It was seen that *F. coffeatum, F. venenatum, F. pseudograminearum* and *F. graminearum* (missing in case of XYL5), belong to an evolved monophyletic group with a significant number of deletions in the amino acid sequences. In comparison to the phyletic outgroup (involving *F. proliferatum, F. fujikuroi* and *F. verticilloides*) they lie in the evolutionary pathway, of the xylanase genes, from *F. oxysporum* sps. to the initially mentioned monophyletic group. This kind of evolutionary grouping can help to understand the relatedness of the toxicogenic potential and hierarchy of evolution of *Fusarium* sps. and better understanding for futuristic use in biocontrol measures.

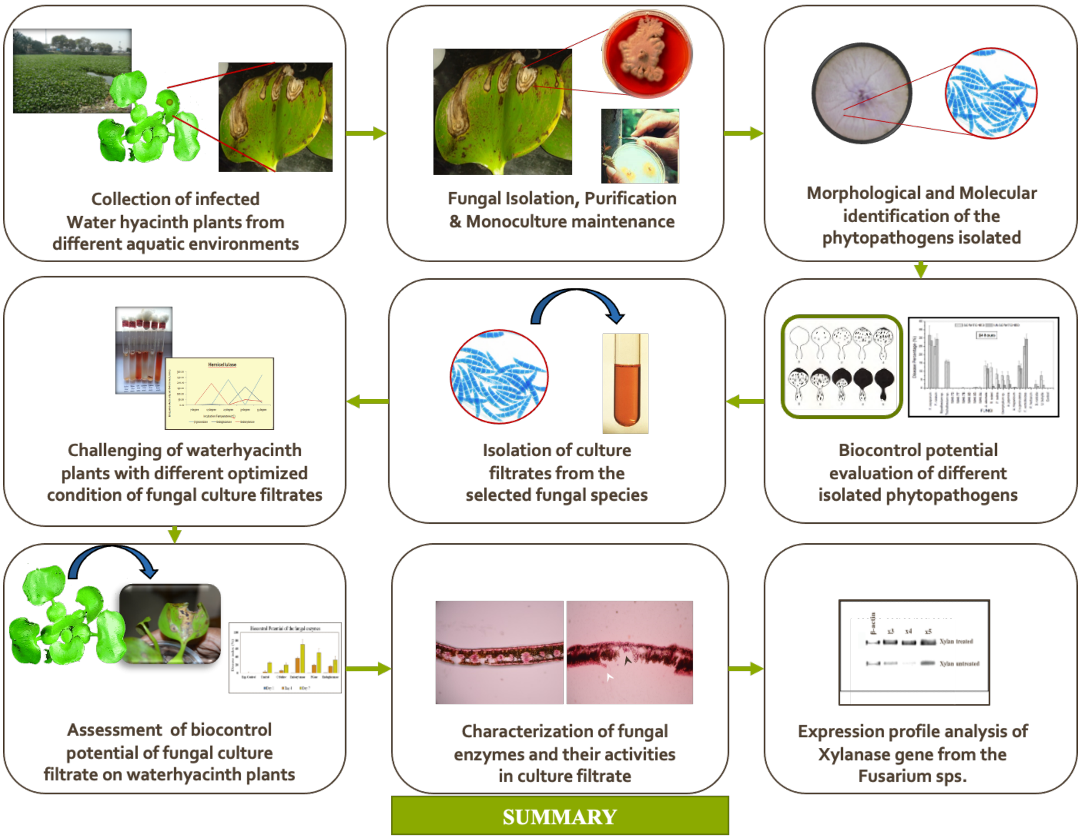

Figure 6.1: Schematic representation of the overall summary of the work done

6.2 Conclusion

In the world of increasing development and application of xenobiotic compounds, such as chemical pesticides, using eco-friendly biological control methods become an absolute necessity to control aquatic weeds without bioaccumulation or leaving a negative impact on the surrounding ecosystem. Using metabolites produced by certain phytopathogenic fungi would be more effective, host-specific and eco-friendly (Saxena and Pandey 2001, Hasan et al. 2021) and utilising the enzymes in weed biocontrol shows much quicker effects in comparison to herbicides and produced by all phytopathogens for as a part of their development phases. With varying environmental conditions in tropical and subtropical regions of the world, where waterhyacinth has invaded, diversity of biological control agents have fluctuated with change in varying conditions (Praveena and Naseema 2006), therefore optimising and characterising the enzymes in order to understand that which would work better under which diverse ecological condition. So, proper understanding and likewise application and improving biocontrol strategies by developing effective mycoherbicides is essential for improving the biocontrol mechanism to work in field conditions. Further the functional analysis of the metabolite or their responsible gene and evolutionary significance helps to predict their future prospects and aspects.

No far earlier reports have documented the use of enzymes or primary metabolites, produced from fungal strains, isolated from the host weeds are being utilized, for the biocontrol of that similar target weed, which in this case is waterhyacinth.

6.3 Future directions and significance

Unlike secondary metabolites produced by selected fungi, all phytopathogens produce primary metabolites, as a part of their growth and developmental process. Several reports have suggested the use of the former in weed-biocontrol. The thesis highlights the use of primary metabolites, in cell wall degradation of the host weed and disintegrating it, playing a pivotal role in its management. The study also redirects to the involvement of the particular gene that play a pivotal role in the degradation process. But here only a particular enzyme from a particular fungi have been carried till the end. Scopes for use of another, or the next in pathogenicity, polygalactonurase could be studied and underlying modes of its action could be investigated, because solely nothing is possible. It is the integration of cumulative effects of various components that together bring about the disintegration of the plant, in this case the weed, waterhyacinth. Not only this work highlights the study from a particular group of fungi, it makes a giant leap in the world of weed biocontrol study, where use of primary metabolites and their modes of actions are prioritized than secondary metabolites which are restricted to only a particular group of selected fungi.

The study broadens the use of more specific eco-friendly means of controlling the aquatic weed, waterhyacinth and at much faster rates by using the primary out products of the fungi, those released during the initial period of the growth phase of the microbe. It aspires in the use of this technology to even detect or understand the change in evolutionary lineages, where mutations occurs in the genes of the amino acid sequences of the enzymes, to adapt to the weed biocontrol mechanism.