Summary and Conclusion

Summary:

- *Fusarium* isolates identified on morphological and microscopical characters in FSSC, FDSC, *F. sacchari* and *F. incarnatum-equiseti* group.
- Molecular identification using ITS resulted in identification of n=18 isolates in FSSC group and n=4 isolates in FDSC group but identification using TEF sequencing showed that n=14 isolates belongs to FSSC, n=3 isolates belongs to FDSC, n=4 isolates belongs to *F. sacchari* and n=1 isolate belongs to *F. incarnatum-equiseti* which was further confirmed in phylogenetic analysis. Hence, TEF is better molecular marker for identification of *Fusarium* spp. and able to discriminate between closely related species.
- Proteases produced by *Fusarium* isolates showed broad range of activity. Optimum pH was 7.4 for maximum number of isolates but protease activity was also high in acidic and basic pH, which indicates that proteases of *Fusarium* spp. are active in broad pH range.
- Inhibition of protease with EDTA, PMSF and Pepstatin A at concentration of 1.0mM, 0.5mM and 1.0μM, respectively did not show inhibition in maximum number of isolates in azocasein assay as well as in gelatine zymography. After increase in inhibitor concentration of EDTA (5mM), PMSF (10mM) and Pepstatin A (10μM), protease activity was inhibited from 20-50%. However, in some of the isolates, inhibition was not seen in gelatine zymography with increased inhibitor concentration. This might indicate stability of *Fusarium* protease at higher concentration of inhibitors.
- Inhibition assay for protease characterization showed that all 3 types of proteases produced by *Fusarium* isolates but serine and metalloprotease are produced by more number of isolates.
- Expression studies were carried out for proteases of FSSC because of availability of WGS database of *N. haematococca*. 14 protease genes, from 50 KDa to 100 KDa molecular weight were selected for expression studies as zymography and SDS-PAGE results showed presence of proteases in this molecular weight range. Only 7 genes

were expressed out of 14 genes in all FSSC isolates. The relative gene expression of C7YY94, C7Z7U2 and C7Z6W1 was maximum in all FSSC isolates in an *in vitro* condition.

- *Ex vivo* infection model showed that *Fusarium* was able to degrade cornea at 10^{th} day. Epithelial layer was degraded by at 2^{nd} day. Fungal mycelia started penetrating in corneal stroma at 4^{th} day and penetrated deep down till 8^{th} day and upon 10^{th} cornea was completely degraded.
- On the basis of specific activity and gene expression studies, isolates selected for an *ex vivo* infection model development and gene expression during an *ex vivo* infection condition were Cc50, CSH3 and Cs1 with lowest, moderate and highest specific activity respectively. Corneal histology at 8^{th} day showed that Cc50 mycelia was less penetrated in corneal stroma and penetration was only on the superficial layer. CSH3 mycelia penetrated in aerial part of cornea but mycelial growth was less compared to Cs1 and Cs1 mycelia penetrated till central as well as near to bottom of corneal stroma and large amount of long hyphae were visible.
- Specific activity in an *ex vivo* infection condition also correlate with *in vitro* data. Maximum activity was seen in Cs1 and minimum activity in Cc50. Gene expression studies showed that C7Z6W1, C7YVF3 were highly expressed in an *ex vivo* infectious condition in all isolates but expression of C7YQJ2, C7Z7U2, C7Z436 and C7ZNV5 was also high in Cs1. Gene expression was highest in Cs1 and lowest in Cc50 during infection.
- C7YVF3 showed maximum expression during an *ex vivo* condition. It also gets expressed during an *in vitro* growth and it was identified in exoproteome analysis of Cs1.
- Protease purification using casein agarose column chromatography showed maximum activity in fraction 15 and also yielded single band in SDS-PAGE. This fraction was identified using MS/MS as Tripeptidyl peptidase.

- Exoproteome analysis of crude extracted protease of Cs1 was carried out and six proteases were identified. These proteases were serine proteases, metalloprotease, carboxypeptidase and aminopeptidase.
- β-glucan extracted from all *Fusarium* isolates showed maximum total β-glucan in FDSC Cc26 and minimum in FSSC Cs1, and maximum β-(1,3)-glucan in FDSC Cc26 and minimum in *F. sacchari* Cc61. Quantification data showed FDSC has more amount of glucan.
- β-glucan characterization showed presence of β-(1,3)-(1,6)-glucan in all isolates except FSSC isolates, CSH1 and Cc50, FDSC isolate Cc119 in which 1—›6 linkage was not found. However, further structural characterization needs to be carried out.
- Immunomodulatory effect of β-glucan extracted from FDSC CSH4 and FSSC CSH5 showed that glucan extracted from CSH4 induced more TNF alpha production. Also mycelia of CSH4 and CSH without NaOH treatment induced equal level of TNF alpha. However, after NaOH treatment, CSH4 mycelia induced more TNF alpha production. NaOH treatment removes upper layer and exposes glucan and this data correlates with quantification data. Also fluorescence microscopy showed more amount of β-(1,3)-glucan in FDSC.
- Gene expression studies showed that G3 and FKS1 are highly expressed followed by GS2 among all glucanses and glucan synthases.
- For toxin and pigment production *Fusarium* isolates were grown in different media and it was found that pigment and toxin production varies depending upon media and isolate.
- ZEA toxin was characterised primarily on TLC. ZEA toxin was identified as fluorescent blue band in presence of U.V. light. HPLC was used for further confirmation and quantification of ZEA toxin in *Fusarium* isolates. Maximum ZEA toxin production was found in Cc61 (*F. sacchari*) and minimum in Cc215 (*F. sacchari*). Further MS/MS showed presence of ZEA toxin in crude extracted toxin which has m/z of 317.
- Pigment production was carried out in PDB, SDB, NB and YMB media and maximum pigment production was seen in PDB and SDB

media. Pigment characterization was also carried out primarily on TLC. The fast orange and slow orange pigment were consistent and were identified as naphthoquinone. For further structural characterization, pigment purification was carried out and characterization of fast orange was done using MS/MS. Two napthoquinone pigments were identified using MS/MS, 3-hydroxy-3-methyl-2-(3,7,11,15-tetramethylhexadec-2-enyl)-2H-naphthalene-1,4-dione and 19-oxoandrost-4-ene-3,17-dione.

Conclusion:

In conclusion, *Fusarium* species produce various types of proteases which are active in wide pH range and are very stable. The requirement of protease production depend upon surrounding environment and hence, certain proteases are highly expressed during *ex vivo* infectious condition and play role during pathogenesis and could be considered as an important virulence factor. *Fusarium* spp. also produce varying amount of β-glucan. The gene expression studies indicated that there is a complex interaction of glucanse and glucan synthase genes as well as the factors which regulates these gens, overall affect the level of β-glucan in *Fusarium* isolates. Immunomodulatory studies revealed that different types of β-glucan are produced by *Fusarium* spp. and there might be variation in the structural conformation of these glucan which further needs to be explored. Also, variation in β-glucan content in cell wall might provide advantage for survival in stressed environment. Similarly, variation in toxin production and pigment production was observed in *Fusarium* spp. Studied of the genes involved in biosynthesis and regulation of toxin and pigment production would be able to provide insight in to these variation. However, toxin and pigment production was not observed in an *ex vivo* infection condition and their role during active *Fusarium* infection is not clear. Secondary metabolites production is reported as important virulence trait of pathogenic fungi but during keratitis their role needs to be investigated. *Ex vivo* infection model can be developed as a potential tool for primary screening of efficacy of drugs during infectious condition.