Chapter 3

Quantification, characterization and expression analysis of extracellular proteases in an *in vitro* condition and corneal explant infection

3.1 Introduction:

Proteases are the enzymes which catalyse hydrolysis of peptide bond (CO-NH) of protein and digesting it into small polypeptide chain or free amino acids. The terms peptidase, proteolytic enzyme, and peptide hydrolase are synonyms of protease (Monod *et al*., 2002; Santos, 2011). Proteases catalyse the reaction at particular pH, temperature and environment. Majority of proteases are sequence specific. (Leung *et al*., 2000; Santos, 2011).

Proteases are well studied molecules which hold important place with respect to their role in various biological processes including physiological as well as infective processes (Naglik *et al*., 2004, 2003; Santos, 2011). Various types of secreted and surface-bound proteases are being produced by pathogenic fungi, which are involved in adhesion, immune evasion, nutrition, invasion, catabolism of host protein, differentiation, cell cycle progression in to the host (Santos, 2011). Variety of functions are being carried out by proteases which varies depending upon cell, organism and organ. It also activate cascade of reactions in haemostasis and inflammation and play important role in normal as well as pathophysiological condition. (Menon and Rao, 2012). Hence it is important to identify and characterise such fungal proteases.

3.2 Materials and Methods:

3.2.1 Growth of fungi:

All isolates were first grown on PDA for active culture. After 7 days of growth, approximately 10 mm disc was cut from the PDA plate of actively growing culture and the disc was inoculated in 100 ml of PDB in 500 ml conical flask (500 ml conical flask was used to provide proper aeration) and allowed to grow in PDB media for 10 days at 30ºC without shaking.

3.2.2 Extraction and precipitation of proteases:

After inoculation of fungus in flask, culture filtrate was aliquoted at Day 2, 4, 6, 8 and 10. The culture filtrate was collected by filtering through a sterile whatman no. 1 filter paper in a sterile bottle. The obtained culture filtrate was subjected to precipitation using chilled acetone. To 1 ml of fungal culture filtrate, 4 ml of chilled acetone was added drop wise. Culture filtrate-acetone mixture was then kept at -20ºC overnight for precipitation. Next day, the

precipitate was collected through centrifugation at 13,000xg for 15 minutes at 4˚C. Resultant precipitate was dissolved in PBS (50 µl PBS/ml of initial culture filtrate used for precipitation) and used for specific activity determination in azocasein assay and protein estimation by Bradford assay.

3.2.3 Protein estimation by Bradford assay:

Protein estimation of concentrated extract was done by Bradford assay. Bovine serum albumin (BSA) (Sigma-Aldrich, USA) was used as a standard at a concentration of 0.1 mg/ml. Bradford reagent was prepared by dissolving 0.010 gm of CBB G-250 (Commasie Brilliant Blue) (Himedia, India) in 5 ml of 95% ethanol. After that, 10 ml of 85% ortho-phosphoric acid (Merck, USA) was added to it and mixed gently and final volume was made 100 ml with D/W. This solution was filtered using whatman no. 1 filter paper and stored in amber coloured bottle at RT and used till 4 weeks but filtered every time when used. The standard BSA concentration range used was 1-10 µg/ml and the volume of standard was set 100 µl with D/W. Sample volume was taken 100 µl and blank was having 100 µl of D/W only. To all the tubes, 1000 µl of Bradford reagent was added and mixed gently and kept at RT for 10 minutes. The OD was measured at 595 nm using spectrophotometer (Multiscan Go, Thermo scientific, USA) and standard graph was plotted and concentration of protein in the sample was plotted from standard graph.

3.2.4 Specific activity measurement by Azocasein assay:

Azocasein assay was carried out by the method of Gifford *et al.,* (2002). Azocasein (Sigma-Aldrich, USA) was dissolved at a concentration of 5 mg/ml in assay buffer containing 50mM Tris-HCL (SRL Chemicals, India) (pH 7.4), 0.2M NaCl (Himedia Laboratories, India), 5mM $CaCl_2$ (Himedia Laboratories, India), 0.05% Brij 35 (Himedia Laboratories, India), and 0.01% sodium azide (Himedia Laboratories, India). The azocasein solution (400 µl) was mixed with 100 µl of precipitated culture supernatants and incubated at 37ºC in a water bath for 90 min. The reactions were stopped by adding 150 µl of 20% trichloroacetic acid (Sigma chemicals, USA), and the reaction mixtures were allowed to stand at the ambient temperature for 30 min. Tubes were then centrifuged for 3 min at 9,000xg. 500 µl of each supernatant was added to 500

µl of 1M NaOH (Himedia Laboratories, India) and allowed to settle for 15 minutes. The absorbance at 436 nm of released azo dye was determined with a spectrophotometer (Multiscan Go, Thermo scientific, USA). One unit of enzyme activity was defined as an increase of 0.1 absorption unit after incubation for 1 hour. Using this definition, the enzyme activity was measured by azocasein assay.

3.2.5 Gelatine zymography:

Zymography was performed by the method described by Lantz and Ciborowski, (1994) using gelatine (Sigma-Aldrich, USA) as a substrate (0.1%). Two glass plates with 0.1mm spacer were used to cast gel. Glass plates were held tightly with clamps on both the sides. 2% agarose solution was used to seal the plates from bottom. After solidification of agarose, 12% SDS-polyacrylamide denaturing gel was prepared using 4 ml of Acrylamide-Bisacrylamide (Mreck, USA) gel stock (30%:0.8%), 2.5 ml of 1.5M Tris (pH 8.8) (Himedia, India), 100 µl of 10% SDS (Merck, USA), 100 µl of 10% APS (Merck, USA) (freshly prepared), 8 µl of TEMED (Merck, USA) and 0.1% Gelatin (Sigma-Aldrich, USA). Final volume was made 10 ml using D/W. 5 ml of this separating gel solution was poured with help of pipette (Thermo scientific, USA) in sealed glass plates. 5% Staking gel was prepared using 330 µl of Acrylamide-Bisacrylamide (Mreck, USA) gel stock (30%:0.8%), 250 µl of 1.0M Tris (pH 6.8) (Himedia, India), 20 µl of 10% SDS (Merck, USA), 20 µl of 10% APS (Merck, USA) (freshly prepared) and 4 µl of TEMED (Merck, USA). Final volume was made 2 ml using D/W. 0.1 mm comb (Merck, USA) was placed in casting plates and separating gel solution was poured. After polymerization, clamps were removed and casting plate was placed in electrophoresis unit and chambers were filled with 1X electrophoresis buffer containing Tris-SDS-Glycine. Combs were removed gently and samples were prepared by mixing with 6X loading dye. Samples were placed in wells slowly and electrophoresis was carried out at 50V initially. When samples entered in separating gel, electrophoresis was carried out at 100V. After electrophoresis, the gel was removed and placed in staining box. Gel was incubated in 2.5% Triton-X 100 (Himedia Laboratories, India) containing incubation buffer (50mM Tris (Himedia, India), 5mM $CaCl_2$ (Merck, USA), 1µM $Zncl_2$

Himedia, India) for 1 hour to remove SDS. After incubation, the gel was washed twice with D/W and incubated in incubation buffer containing at 37˚C for 2 hours/overnight if required. After that, the gel was again washed with D/W and kept overnight for staining with (0.5%) CBB R 250 (Himedia Laboratories, India) prepared in Methanol (Merck, USA): Water: Acetic Acid (Merck, USA) (4:5:1) (V/V). Next day, the stained gel was de-stained with Methanol: Water: Acetic acid (4:5:1) (V/V) solution to observe the bands of enzyme activity. The bands of substrate degradation were observed as colourless bands against blue background.

3.2.6 SDS-PAGE (Sodium Dodecyl sulphate-Polyacrylamide Gel Electrophoresis):

15% SDS- polyacrylamide gel was prepared as following. Two glass plates with 0.1mm spacer were used to cast gel. Glass plates were held tightly with clamps on both the sides. 2% agarose solution was used to seal the plates from bottom. 15% SDS-polyacrylamide denaturing gel was prepared using 5 ml of Acrylamide-Bisacrylamide (Mreck, USA) gel stock (30%:0.8%), 2.5 ml of 1.5M Tris (pH 8.8) (Himedia, India), 100 µl of 10% SDS (Merck, USA), 100 µl of 10% APS (Merck, USA) (freshly prepared) and 8 µl of TEMED (Merck, USA). Final volume was made 10 ml using D/W. 5 ml of this separating gel solution was poured in sealed glass plates. 5% Staking gel was prepared using 330 µl of Acrylamide-Bisacrylamide (Mreck, USA) gel stock (30%:0.8%), 250 µl of 1.0M Tris (pH 6.8) (Himedia, India), 20 µl of 10% SDS (Merck, USA), 20 µl of 10% APS (Merck, USA) (freshly prepared) and 4 µl of TEMED (Merck, USA). Final volume was made 2 ml using D/W. 0.1 mm comb (Merck, USA) was placed in casting plates and separating gel solution was poured in sealed glass plate. After that, clamps were removed and casting plate was placed in electrophoresis unit and chambers were filled with 1X electrophoresis buffer containing Tris-SDS-Glycine. Combs were removed gently and samples were prepared by mixing with 6X loading dye. Samples were placed in wells slowly and electrophoresis was carried out at 50V initially. After samples crossed stacking gel, electrophoresis was carried out at 100V. After electrophoresis, the gel was removed and placed in staining box, washed with D/W and kept overnight for staining with (0.5%) CBB R 250

(Himedia Laboratories, India) prepared in Methanol (Merck, USA): Water: Acetic Acid (Merck, USA) (4:5:1) (V/V). Next day, the stained gel was de-stained with Methanol: Water: Acetic acid (4:5:1) (V/V) solution to observe the blue bands of protein against colourless background.

3.2.7 Characterization of proteases:

For characterization of proteases, the effect of pH and inhibitors was studied on crude proteases extracted from all fungal isolates.

3.2.7.1 Effect of pH on proteases:

The effect of pH on enzyme activity was studied at 5 different pH by incubating the crude enzyme extract in azocasein solution of pH 3, pH 5, pH 7.4, pH 8 and pH 10. Azocasein assay was carried out using standard protocol and absorbance was measured at 436 nm in spectrophotometer (Multiscan Go, Thermo scientific, USA). One azocasein unit is defined as an increase of 0.1 absorption unit after incubation for 1 hour. Using this definition, the enzyme activity was measured by azocasein assay.

3.2.7.2 Effect of enzyme inhibitors on proteases:

To understand the type of enzyme produced by fungal isolates, specific enzyme inhibitors were used and azocasein assay was carried out. PMSF (Sigma-Aldrich, USA) (inhibitor of serine protease at final concentration of 5.0mM), EDTA (Himedia, India) (inhibitor of metalloprotease at final concentration of 10mM) and Pepstatin A (Sigma-Aldrich, USA) (inhibitor of aspartyl proteases at final concentration 10µM) were used to check the type of proteases present in fungal isolates. Enzyme samples were incubated with inhibitor for 1 hour and same concentration of inhibitor was also added in azocasein solution in which the azocasein assay was carried out. Azocasein assay was carried out using standard protocol and absorbance was measured at 436 nm in spectrophotometer (Multiscan Go, Thermo scientific, USA). One azocasein unit is defined as an increase of 0.1 absorption unit after incubation for 1 hour. Using this definition, the enzyme activity was measured by azocasein assay. The activity of control proteases (without inhibitor) was compared with inhibitors treated proteases. The control activity was

considered as 100% and with respect to that %residual activity was calculated for samples.

3.2.7.3 Zymography in presence of inhibitors:

Zymography was performed in presence of inhibitors also. The proteases samples were pre-incubated with three inhibitors, PMSF at final concentration of 5.0mM, EDTA at final concentration of 10mM, and Pepstatin A at final concentration of 10µM for 1 hour. The samples were mixed with loading dye and gel was prepared as mentioned earlier and then loaded into gel. Zymograpy was carried out by standard method as mentioned earlier.

3.2.8 LCMS-MS (Liquid chromatography- Mass spectroscopy):

3.2.8.1 Sample preparation:

Crude enzyme extract was concentrated using 10 KDa M.W. cut-off column (Merck, USA). Protein concentration of obtained crude extract was measured using Bradford assay. 50 µg of sample was taken and final volume was made up to 10 µl with 25mM ammonium bicarbonate buffer. Then 20mM Dithotheritol (DTT) was added to carry out reduction. The sample was incubated at 56ºC for 1 hour in dry bath. Alkylation was carried out using Iodoacetamide and samples were incubated at RT for 30 minutes. After alkylation, sample was digested with trypsin in ratio of 1:30. The sample was incubated at 37ºC for 16-18 hours. The sample was dried under vacuum. To the dried sample, 20 µl of 0.1% formic acid was added. Sample was de-slated with 50% acetonitrile (ACN), followed by 80% ACN. C18 reverse phase (RP) column was activated by using 100% ACN. Column was washed with 0.1% formic acid. The sample was solubilised in 0.1% formic acid and passed through the column. Column was eluted using 50% ACN, followed by 80% ACN. The obtained fraction was then dried under vacuum. The dried sample was re-suspended in 0.1% formic acid and used for MS/MS analysis using Q-Exactive Plus Biopharma-High Resolution Orbitrap (Thermo scientific, USA).

3.2.8.2 MS/MS parameters:

A capillary column **(**C18, 2 µm, 100 Å, 75 µm×50 cm or 15 cm Thermo scientific, USA) was used for separation of peptides. Sample was first loaded on a pre-column (Acclaim® PepMap 100, C18, 3 µm particle size, 100 Å, 75

μm×2 cm Thermo scientific, USA) from an auto-sampler at maximum pressure of 1000 bar. Following the pre-column, the sample was then analysed using an analytical column with a linear gradient program where the components of solution were changed from 5% B to 100% B over 90 min at a constant flow rate of 300 nl/min (95% ACN in 0.1% FA from 5% to 10% over 5 min, 10% to 50% over 45 min, 50% to 95% over 5 min and kept at 100% for 5 min at a flow rate of 300 nl/min). Positive mode electrospray ionization was used. Full scan profile mass spectra were acquired over m/z of 350-2000 Da at a frequency of 1 spectrum every sec.

3.8.2.3 Data analysis:

The raw data was analysed using Proteome Discoverer 2.1 (Thermo scientific, USA) using Mascot (Matrix Science, London, UK; version 2.4.1.0) and the inbuilt SequestHT search algorithm. The database search was performed with the following parameters: peptide tolerance of 10 ppm and fragment tolerance of 0.60 Da to 0.80 Da, allowing two missed cleavages. Fixed modification was given as cysteine carbamidomethylation and variable modifications were methionine oxidation, N-terminal acetylation and phosphorylation (S, T, and Y). The peptide spectrum matches (PSMs) from SequestHT and Mascot were post-processed using the percolator algorithm.

3.2.9 RNA isolation:

Fungal isolates were grown in PDB at 30ºC for 7 days and mycelial mat was collected by filtration using whatman no. 1 filter paper (Merck, USA). Fungal mat was washed with D/W and dried by decanting the water. 60-70 mg of dried mat was taken in to sterile mortar pestle and 1 ml of RNA iso plus reagent (TAKARA, Japan) was added and tissue was grinded in mortar pestle and collected in to sterile diethyl pyrocarbonate (DEPC) (MP Biochemicals, India) treated MCT. Tubes were kept at RT for 5 minutes. After incubation, 0.2 ml of chloroform was added in to it and tubes were shacked for 15-30 seconds and kept at RT for 2-3 minutes followed by centrifugation at 4ºC for 15 minutes at 12,000xg. After centrifugation, 3 layers were separated and uppermost layer which contained RNA was taken in to sterile DEPC treated MCT. For 1 ml of RNA iso plus reagent, 0.5 ml of isopropanol (Merck, USA)

was added and tube was inverted slowly to mix the contents and kept at RT for 10-15 minutes. After incubation, centrifugation was carried out at 12,000xg at 4ºC for 10 minutes. After centrifugation, supernatant was discarded and pellet was washed with 70% ethanol twice followed by centrifugation at 7,500xg at 4ºC for 5 minutes. The pellet was air dried for 5-10 minutes and dissolved in nuclease free water and stored at -20ºC till further use. Quality of RNA was analysed by agarose gel electrophoresis using 2% agarose gel and by taking ratio of 260/280 nm and quantification was done by measuring absorbance at 260 nm using spectrophotometer (Multiscan Go, Thermo scientific, USA). After quantification, RNA was treated with DNase (Rosche, USA) to remove possible DNA contamination. For that 10 µg RNA was taken in to DEPC treated sterile MCT. 3 units of DNase and 3 µl of DNase buffer was added and final volume was made 25 µl with dH_2O. This mixture was incubated at 37ºC for 10 minutes. After incubation, 0.2M EDTA was added to block the enzyme and enzyme was deactivated by incubation at 75ºC for 10 minutes in dry bath (Genei, India). After deactivation of enzyme, 1 ml chilled isopropanol was added to it and kept at -20ºC for overnight. Next day, centrifugation was carried out at 4ºC at 12,000xg for 10 minutes. Supernatant was discarded and pellet was washed twice with 70% ethanol followed by centrifugation at 7,500xg for 5 minutes. Pellet was air dried and dissolved in nuclease free water. Quality assessment and quantification was carried out as mentioned earlier. Once RNA was free of DNA contamination it was stored at -20ºC till further use.

3.2.10 Complementary DNA (cDNA) synthesis:

For cDNA synthesis, Primerscript 1st strand cDNA synthesis kit (TAKARA, Japan) was used. Kit was provided with random 6-mer primer, oligo dT primer, dNTPs, 5X primer script buffer, Reverse transcriptase, RNase inhibitor and nuclease free water. First of all, 5 µg of RNA was taken as template. 2 µl of random 6-mer primer, 1 µl of oligo dT primer and 1 µl of dNTPs were added to it. Final volume was made 10 µl with nuclease free water. The reaction was carried out in PCR thermocycler (BIO-RAD, USA). PCR reaction involved incubation at 30ºC for 10 minutes followed by 65ºC for 5 minutes. After that, the reaction was stopped and PCR tube was placed on

ice for 5 minutes followed by addition of 4 µl of 5X primer script buffer, 1 µl of reverse transcriptase and 0.5 µl of RNase inhibitor. The final volume was made up 20 µl with nuclease free water. The PCR reaction was carried out as follows: 50ºC for 1 hour, 75ºC for 15 minutes and halt at 4ºC. The cDNA was stored at -20ºC till further use.

3.2.11 Primer designing of protease genes:

Primer designing for protease gene was done for *Nectria haematococca* only, as whole genome data is available for *N. haemoatococca*. First proteases were screened for excretory signal using Signal P 4.0 followed by their molecular weight and then their function/activity. UniProt KB was used to find out proteases present in *N. haematococca*. Only those proteases which gave positive secretory signal were included in study. 14 proteases, 10 high molecular weight and 4 low molecular weight proteases were selected. The gene sequences for these proteases were obtained from Ensemble Fungi and NCBI. As housekeeping gene, translation elongation factor 1 alpha (TEF1-alpha) was used. Primers were designed using primer designing tool, NCBI Primer BLAST. The quality and properties of primers were checked using OligoCalc tool. Primers were synthesised commercially by Eurofins, India (Table 3.1).

3.2.12 Real Time PCR (qPCR):

Real time PCR was carried out using BIO-RAD CFX Manager thermocycler. cDNA was used as template. SyBr Green premix (TAKARA, Japan) was used as fluorescent dye for real time analysis. For amplification of protease gene, respective primers were added in to PCR reaction mixture. Reaction contained 2 µl of cDNA template, 10 pmol of forward and reverse primer of respective gene, 10 µl of SyBR premix and final volume was set 20 µl using deionized water (dH_2O). Reaction mixture was added to real time PCR plates (Thermo Scientific, USA) and plates were sealed with sealant (Thermo Scientific, USA). The PCR reaction involved following steps: 1 cycle of 95ºC for 3 minutes, 40 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds. Plate read was carried out at the end of each cycle. Final extension was carried out at 72ºC for 5 Minutes. Melt curve was set at 65ºC-95ºC with

increment of 0.5ºC for 5 seconds followed by plate read at every step. Relative gene expression was calculated using $2^{\wedge(-\Delta\Delta CT)}$.

3.2.13 Development of an *ex vivo* infection model for fungal keratitis and expression analysis of fungal virulence factors in different phases of infection.

3.2.13.1 *Ex vivo* caprine (goat) cornea culture:

Collection of goat eye balls:

Goat eye balls were collected from slaughter house in sterile beakers.

Sterilization of goat eye balls: Eye balls were washed five times with 1X Phosphate buffer saline (PBS) and extra tissue was removed with the help of sterile scissors. Eye ball was then kept in 2.5% povidone iodine solution (Cipla, India) for 5 minutes. Eye balls were then taken in to another sterile beaker and washed three times with sterile 1X PBS containing 0.2 mg/ml gentamycin, 100 I.U./ml penicillin (Thermo scientific, USA) and 100 µg/ml streptomycin (Thermo scientific, USA). Then eye balls were kept in 0.1% gentamicin (Pfizer, India) for 15 minutes. After that, again eye balls were washed three times with 1X PBS containing gentamycin, penicillin and streptomycin as mentioned above.

Dissection of cornea and explant culture of cornea: Eye balls were taken in to sterile paper towel and held in such a way that cornea remains upward. Sterile scalpel was used to make an incision in the limbal region of the eye ball. After making incision, aqueous humor started coming out and sterile scissors were used to further expand the incision to cut around the middle of the eye, cutting the eye in half. After separation of cornea from rest of the eye, it was kept in sterile glass petri plates containing 1X PBS with antibiotics as mentioned above. Corneas were washed three times with 1X PBS containing antibiotics. These corneas were then cultured. For culturing corneas, artificial beads/corneal buttons were prepared. For preparation of artificial corneal buttons, 0.5% agarose and 0.5% gelatine were prepared in 1X PBS and autoclaved separately and then mixed.

Table 3.1: The genes investigated in current study, their known function and primers used for qRT-PCR.

No	Uniprot ID	MW (kDa)	Putative function	Primer sequences (5'—›3')
1	C7Z0E6	95.93	serine-type endopeptidase activity	F:CTGGCGCAGGGAGGTAA R:TGGTTGAGGAGCGATATCCAT
2	C7ZFW9	97.81	serine-type endopeptidase activity	F:CGAGTTTGAGGATGGAACCAA R:ATTCCTCCTCCTGGAGGGTAT
3	C7Z7U2	94.29	serine-type endopeptidase activity	F:GTTGACAAGTTGCGAGCTGA R:TGCGTTCTTGCCGTTGTA
4	C7ZNV5	92.61	serine-type endopeptidase activity	F:AAGGCCGAAGGTTATGTCGA R:ATGGCTTCACCCCACAGCTT
5	C7YY94	94.32	serine-type endopeptidase activity	F:TGCTGGAGAATCTTGATGCT R:TTGCCCTCTTTTAGAAGGGA
6	C7Z7Y4	78.37	Palmitoyl transferase activity	F:TGGGCCGCAATTAACAACCAG R:GGTGGAGAAGAAGCACGAG
7	C7YQJ2	60.16	serine-type carboxypeptidase activity	F:GTGCTGGGCGCTGCGTCGT R:ACGTGATCCCAGTGGTTGT
8	C7YVF3	62.13	serine-type carboxypeptidase activity	F:CCAGGCAGCTTTCAACCGGG R:CTCTGGACTCATCCAGGGT

9	C7YPA2	82.66	serine-type endopeptidase activity	F:GTTTCAAAGAGTTCGAGTGC R:GACAGTGGCACCTTTGCCTCGT
10	C7Z8P9	65.65	serine-type endopeptidase activity	F:ATGAAAGCGATATCACTCA R:GGCTTCCTTCTGGGTGA
11	C7YSA1	66.47	serine-type endopeptidase activity	F:ATGCGGCCTTTGCTCGCGCTGA R:GCAAGACCTGTCGTGGTTGA
12	C7Z436	78.97	serine-type peptidase activity	F:ATGCGGCCTTTGCTCGCGCTGA R:GTCCTTGAGGGCGTCGACTGTAGA
13	C7YSV9	56.83	serine-type endopeptidase activity	F:GCCGTTTCTGAGTACAAGTGC R:GTTGCCATCCTCATCAGCATCGC
14	C7Z6W1	43.54	Aminopeptidase activity	F:AAGAACCTCGAGAAGAAGAAC R:CATCGTCATCGGCGCCAGGAGC
15	TEF1 alpha	F: TTCAAGTGGGCGATGCTCTT R: AGTTGATGGGGTCTGCTGTG		

Simultaneously surface sterilised parafilm was placed on to sterile glass tube and starched/excavated inside the tube. The dimensions of the parafilm were 4x4 inches and the surface sterilization was done using 70% alcohol for two hours. The warm agarose-gelatine solution was poured in the excavated parafilm and allowed to cool. Upon cooling, the agarose-gelatine formed the shape of a concave bead. These beads were placed in to sterile 12 well tissue culture plate (Tarson, India). The dissected corneas were placed on these artificial beads in such a way that the endothelial layer remained in direct contact with bead, while epithelial layer facing air and then 1 ml of DMEM containing 10% FBS (Hyclone, USA), penicillin (100I.U/ml), streptomycin (75µg/ml) and gentamycin (35 µg/ml) was added to each well. The plate was incubated at 37ºC at 5% CO_2 in CO_2 incubator (Thermo Fisher, USA). Media was changed after every 24 hours. The corneas were then observed for the required time interval and tested for viability. Later on, the agarose-gelatine beads were replaced with 1% agarose beads made in DMEM medium. For this, 2% agarose solution was made in 1X PBS and autoclaved and then mixed with equal amount of DMEM media. Also preparation of beads with help of parafilm was replaced with hollow semicircle stainless steel beads which were autoclavable.

3.2.13.2 Cornea viability assay:

Viability of corneal epithelial cells was checked using trypan blue assay; while viability of entire cornea was done using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay.

3.2.13.2.1 MTT assay:

Whole cornea was used for MTT assay. At required time interval MTT assay was done using EZcount TM MTT cell Assay kit (Himedia laboratories, India) following manufacturer's protocol.

3.2.13.2.2 Trypan blue assay:

The trypan blue assay was done after separation of epithelial cells. Separation of epithelial cells was done using trypsin treatment. The cornea was treated with 0.25% of trypsin (Gibco, Thermo scientific, USA) for 12 hours at 37ºC. After trypsinization, cornea was washed and placed in 1X PBS and with the

help of scalpel blade, the upper half of the cornea was scratched gently. The scratching removes the epithelial cells in the PBS. The PBS with cells was taken in a MCT and centrifuged at 6000 rpm at 4ºC for 20 minutes. The supernatant was discarded and the cells were re-suspended in 100 µl of 1X PBS. 100 µl of 0.4% trypan blue prepared in 1X PBS was added to the above cell suspension and incubated for three minutes. The cell suspension was loaded on a hemocytometer and examined immediately under a microscope. The number of stained cells (dead cells) and the total number of cells were counted manually and the viability was calculated using the formula: % of viable cells = Number of viable cells x 100/Total number of cells.

3.2.13.3 Infection of cornea with *Fusarium solani:*

The corneas were sterilised and explant culture was developed as mentioned above and before placing corneas on to artificial beads corneal surface was gently scratched with sharp pointed scalpel. These corneas were washed and placed on artificial beads and were infected with the help of sterile wire loop or sterile tip with spores of *F. solani* in triplicates. *F. solani* was grown on PDA for 8-10 days and spores were harvested by adding 5-7 ml of sterile 1X PBS in to the plate followed by centrifugation at 5000 rpm at RT. The pelleted spores were re-suspended in 1 ml of 1X PBS and spores were counted using haemocytometer. 10^6 spores were used initially then 10^4 spores were used and later all experiments were done using 932±46 spores as well as 10^4 spores. Presence of *F. solani* on the infected cornea and the culture medium was checked at regular time points by streaking on PDA plates as well as NA plates. Media was changed every 24 hours initially, then every 12 hours, and then every six hours. The progression of infection was monitored everyday visually as well as by keratome sectioning followed by Haemolysin-Eosin (H-E) staining. Specimens for histology (infected cornea) were fixed in 2% buffered paraformaldehyde for 15 minutes. The paraffin embedded samples were sectioned at a thickness of 5 µm. Sections were taken on silane coated slides and deparaffinized slides were processed for H-E stain. Stained sections were mounted in DPX (distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene) and imaged using Leica DM 750 light microscope and LasEZ software (Leica Microsystems, India).

3.2.13.4 Specific activity measurement by azocasein assay and protein estimation by Bradford assay:

After infection of cornea, media was changed at every six hours and taken in to sterile MCT. This media was used for specific activity determination and protein estimation using azocasein assay and Bradford assay.

3.2.13.5 Quantitative RT-PCR (qRT-PCR):

Fungal infected corneas were harvested at 2nd, 4th and 8th day initially. RNA isolation, cDNA synthesis and real time PCR were carried out as mentioned above. Maximum fold change was seen at 8th day so later on fungal infected corneas were harvested at 8th day. Real time PCR was performed for all protease genes mentioned earlier.

3.2.14 Statistical analysis:

All the analysis were conducted using Graph Pad Prism 6.01. Results are given as mean values and standard deviation of the same were calculated. Tests applied on data were ANOVA, Fisher's LSD, Brown-Forsythe test, Dunnett's test and Holm-Sidak test. Statistically significant difference was considered at p value < 0.05.

3.3 Results:

3.3.1 Specific activity of proteases:

The specific activity was calculated in terms of units/mg of protein. Among isolates of *Fusarium* spp., specific activity varies. The maximum activity was observed in FSSC isolate Cs1 which was 223.68±18.83 units/mg and the minimum activity was observed in *F.sacchari* isolate Cc52 24.02±0.56units/mg. Table 3.2 shows specific activity of all *Fusarium* isolates at pH 7.4. The values are represented as mean±SD. In FSSC isolates, the specific activity was ranging from 30.05±0.73 units/mg (Cc50) to 223.68±18.83 units/mg (Cs1). In FDSC isolates, Cc26, Cc119 and CSH4 has specific activity of 37.65±1.92 units/mg, 42.24±0.24 units/mg and 154.15±0.80 units/mg, respectively. In *F. sacchari* isolates, specific activity was 24.02±0.56 units/mg, 33.35±0.54 units/mg, 158.90±3.18 units/mg and108.15±7.77 units/mg in Cc52, Cc61, Cc167 and Cc215, respectively. *F. equiseti* isolate was having specific activity of 173.70±5.25 units/mg. The

statistically significant difference was considered when *p*-value was less than 0.05.

3.3.2 Protease characterization:

For characterization of proteases, the effect of pH and enzyme inhibitors were studied on crude acetone precipitated enzyme extract from all *Fusarium* isolates using azocasein assay and gelatine zymography.

3.3.2.1 Effect of pH:

pH range used for protease characterization was pH 3.0, pH 5.0 pH 7.4, pH 8.0 and pH 10 to find optimum specific activity in each isolate. Protease from *Fusarium* spp. showed activity at all pH tested which indicates that protease was active in broad pH range. Table 3.2 shows specific activity of all *Fusarium* isolates at all five different pH. The values are represented as mean±SD. Among all the isolates of *Fusarium* spp., the optimum pH observed was pH7.4 except for few isolates. *F. sacchari* isolate Cc52 and FSSC isolate Cc50 was having optimum pH of 3.0 with specific activity of 38.34±7.32 units/mg and 30.43±0.0 units/mg, respectively. In some of the isolates there was very less difference seen among all pH, for e.g. Cs2 but statistically difference was considered significant by ANOVA (Analysis of Variance), Holm-sidak's test and Fisher's LSD (Least significant Difference). FSSC isolate Cc240 showed similar activity at all five pH range and no statistical difference was observed. This result indicates that optimum pH for *Fusarium* isolates, is pH 7.4 and are neutral protease but the protease is active in acidic and alkaline environment also and can be stable in broad pH range.

Table 3.2: Specific activity of extracted proteases from *Fusarium* spp. at different pH using Azocasein assay.

	pH 3.0	pH 5.0	pH 7.4	pH 8.0	pH 10.0
FSSC isolates					
CSH 1	123.29±2.13	132.17±1.77	163.93±4.70	53.96±2.37	126.71±7.69
CSH 2	115.83±2.01	113.48±3.81	166.19±4.62	39.28±5.23	109.45±1.16
CSH 3	165.50±9.06	125.63±5.32	199.68±12.49	100.17±10.84	149.09±5.72
CSH 5	146.81±3.23	151.98±9.30	215.04±8.95	43.42±7.11	147.32±3.10
CSH 6	107.06±3.45	112.72±4.59	165.82±2.61	18.28±1.31	110.11±3.99
CSH 7	134.24±12.94	123.39±10.85	185.77±22.20	22.15±3.41	124.30±2.07
CSH 8	140.40±2.41	133.80±4.70	164.38±9.63	131.02±0.00	131.02±4.70
CSH 9	51.22±1.89	53.94±0.00	95.35±8.06	49.04±1.63	40.86±3.27
CSH 10	91.58±1.53	94.23±6.24	130.33±5.34	84.54±3.49	76.17±2.02
CSH 11	89.44±4.24	90.09±3.13	111.15±3.93	82.31±2.81	80.69±5.91
CS 1	199.86±3.91	207.62±9.99	223.68±18.83	192.09±3.23	154.29±0.90

Cs 2	154.76±2.44	166.54±4.92	176.29±3.72	151.10±3.22	154.76±2.44
Cc50	38.34±7.32	32.12±0.73	30.05±0.73	27.98±2.19	29.01±3.66
Cc240	95.87±0.0	94.66±4.08	93.94±1.02	92.74±0.68	92.01±1.70
FDSC isolates					
Cc 26	20.46±1.15	19.64±0.38	37.65±1.92	26.19±0.38	20.19±1.15
Cc 119	15.84±2.16	17.03±0.72	42.24±0.24	13.28±0.24	14.30±0.24
CSH 4	120.70±5.47	126.06±3.50	154.15±0.80	55.67±3.24	114.82±2.12
***F. sacchari* isolates**					
Cc 52	30.43±0.0	28.43±1.13	24.02±0.56	19.22±0.56	22.82±2.26
Cc 61	24.43±0.54	22.49±0.54	33.35±0.54	20.55±1.09	17.84±3.83
Cc167	115.16±4.56	143.65±2.51	158.90±3.18	105.53±0.69	99.91±3.18
Cc215	46.20±4.81	59.32±0.91	108.15±7.77	50.92±0.91	36.75±1.82
F. incarnatum-equiseti					
Cc 172	148.43±4.69	158.54±3.89	173.70±5.25	131.25±2.59	127.35±4.68

3.3.2.2 Effect of inhibitors:

To characterize the protease, in the present study 3 different inhibitors were used. The inhibitors used were PMSF which is the inhibitor of serine proteases, EDTA which inhibits the activity of metalloproteases and Pepstatin A which inhibits the activity of aspartyl proteases. The inhibitor concentration used was 1.0mM, 0.5mM and 1.0µM of inhibitors EDTA, PMSF and Pepstain A. The above mentioned concentration of inhibitors did not show good inhibition of protease. The % inhibition was found up to 10-15% only. This indicates the stability and diversity of protease being produced by *Fusarium* spp. After that, the inhibitor concentration was increased up to 10 times and the azocasein assay in presence of inhibitors was again carried out and % residual activity was calculated. The new inhibitor concentrations used were 10.0mM, 5.0mM and 10.0µM of EDTA, PMSF and Pepstatin A, respectively. Table 3.3 shows the % residual activity of proteases from *Fusarium* isolates in presence of inhibitors. When concentration was increased, the protease activity was inhibited up to 70-80% in some of the *Fusarium* isolates and up to 50% in some of the isolates. However in presence of inhibitors, the protease activity of some of the isolates was increased. The maximum increase was seen in case of PMSF. The reason of such increase in activity in presence of inhibitor is still not clear. The *Fusarium* isolates were getting inhibited by all 3 types of proteases. The protease activity of FSSC isolates was inhibited by EDTA majorly. Protease activity of isolates, CSH1, CSH2, CSH5, CSH6, CSH8, CSH9, CSH10 and CSH11 was inhibited by EDTA. Protease activity of isolates, CSH5, CSH6, CSH7 and CSH11 was inhibited by PMSF. Protease activity of isolates, CSH1, CSH2, CSH6, CSH9, CSH11, Cs1, Cs2 and Cc50 was inhibited by Pepstatin A. These results indicates that FSSC isolates produce more than one type of protease. In *F. delphenoides* isolates the inhibition of protease activity was seen in presence of all 3 inhibitors. However more inhibition was seen in presence of EDTA. In *F. sacchari* isolates, the protease activity was also inhibited by all 3 inhibitors. Isolates majorly showed inhibition of protease activity with Pepstatin A. Protease activity of *F. equiseti* isolate Cc172 was also inhibited by all 3 inhibitors but maximum inhibition was seen in presence of PMSF.

Table 3.3: % residual activity of proteases in presence of inhibitors (EDTA, PMSF and Pepstatin A) by using azocasein assay.

Isolate	% Residual activity					
	EDTA (1.0mM)	**EDTA (10.0mM)**	**PMSF (0.5mM)**	**PMSF (5.0mM)**	**Pepstatin A (1.0µM)**	**Pepstatin A (10.0µM)**
			FSSC isolates			
CSH1	105.90±2.84	73.86 ± 6.25	90.82±1.11	94.31 ± 12.90	87.44±5.44	79.16 ± 3.28
CSH2	77.27±2.00	80.57 ± 1.89	95.97±13.76	108.26 ± 5.85	94.35±4.03	85.12 ± 3.98
CSH3	104.17±6.62	131.22 ± 3.69	89.08±12.16	104.91 ± 1.21	76.82±1.66	91.92 ± 5.79
CSH5	106.20±6.61	79.62 ± 1.60	89.29±9.52	87.03 ± 3.20	117.86±4.29	94.44 ± 2.77
CSH6	108.12±7.83	71.57 ± 1.75	85.37±13.32	73.09 ± 2.63	99.66±7.93	88.83 ± 2.32
CSH7	102.68±1.33	93.86 ± 4.25	87.21±3.08	87.11 ± 2.81	101.93±2.42	95.09 ± 2.12
CSH8	92.59±0.00	76.35 ± 2.05	134.45±11.37	145.10 ± 2.44	128.85±21.93	120.38 ± 3.29
CSH9	91.92±3.50	59.77 ± 7.17	283.33±34.42	145.97 ± 5.26	385.42±31.46	75.86 ± 8.67
CSH10	96.35±0.90	70.58 ± 5.63	104.37±20.07	128.87 ± 2.45	124.21±3.00	97.86 ± 0.92
CSH11	92.94±2.35	42.26 ± 1.19	118.56±25.14	79.38 ± 7.01	116.67±16.75	69.07 ± 1.19

Cs1	97.67±3.55	93.93 ± 3.14	109.18±5.10	152.12 ± 7.92	138.44±3.86	83.63 ± 2.77
Cs2	97.22±3.58	87.30 ± 3.51	108.12±10.73	113.19 ± 6.15	119.42±8.03	83.75 ± 7.51
Cc50	81.33±3.77	81.33±3.77	84±1.88	84±1.88	81.33±3.77	54.76 ± 7.14
Cc240	96.92±0.36	96.92±0.36	98.46±0.72	98.46±0.72	99.48±0.36	99.48±0.36
FDSC isolates						
Cc26	83.26±3.35	46.85 ± 4.42	204.52±20.6	56.52 ± 2.21	101.91±4.17	59.90 ± 9.20
Cc119	45.00±0.55	28.49 ± 1.23	116.34±0.0	49.73 ± 4.65	80.76±0.55	33.60 ± 4.90
CSH4	99.52±3.59	78.88 ± 3.20	128.25±3.12	93.70 ± 1.11	89.22±0.35	84.81 ± 3.57
***F. sacchari* isolates**						
Cc52	84.12±2.46	253.33 ± 7.69	160.14±0.0	111.11 ± 6.66	92.17±4.31	63.33 ± 3.84
Cc61	73.10±0.57	131.78 ± 2.32	136.31±9.85	68.99 ± 5.85	86.23±4.33	44.18 ± 2.68
Cc167	82.82±3.49	64.53 ± 1.00	80.42±5.53	84.30 ± 3.02	125.38±6.50	77.90 ± 5.23
Cc215	81.98±3.12	59.57 ± 3.68	85.41±9.54	93.61 ± 9.75	178.12±29.81	53.19 ± 7.37
***F. incarnatum-equiseti* isolate**						
Cc172	85.62±1.94	75.61±5.58	78.29±3.10	64.02±6.42	95.67±2.48	71.95±2.79

3.3.2.3 Gelatine zymography:

Gelatine zymography was performed using 0.1% gelatine in 12% SDS-PAGE. The crude enzyme extract which was used for azocasein assay was also used for gelatine zymography. The 0.2 units of enzyme (calculated from specific activity estimation using azocasein assay) was incubated with inhibitors for 1 hour and control sample was incubated without inhibitors and then loaded in to the gel. The zymography was performed with initial inhibitor concentration of 1.0mM, 0.5mM and 1.0μM concentration of EDTA, PMSF and Pepstatin A, respectively. However, no inhibition was detected in zymography gel. So inhibitor concentration was increased 10 time similar to azocasein assay. The new inhibitor concentration was 10.0mM, 5.0mM and 10.0μM for EDTA, PMSF and Pepstatin A, respectively.

Crude protease of *Fusarium* species showed various type of banding pattern in zymography. Crude extracted protease of some of the isolates was not inhibited by any inhibitors but crude protease of some isolates was inhibited with one or two inhibitors. In FSSC, crude protease of CSH1 (Figure 3.1 A), CSH9 (Figure 3.2 B), CSH10 (Figure 3.2 C), Cs1 (Figure 3.2 E), Cs2 (Figure 3.2 F) and Cc240 ((Figure 3.3 B) did not show inhibition with any of the inhibitors. Lane with inhibitors were of same intensity of control lane.

The crude protease of CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH11 and Cc50 was showing inhibition in presence of selected inhibitors. Crude protease of CSH2 (Figure 3.1 B), showed inhibition with EDTA and PMSF. The intensity of bands were very faint compared to control lane but it did not show inhibition with Pepstatin A. Crude protease of CSH3 (Figure 3.1 C), also showed inhibition with EDTA and PMSF as the intensity of lanes with inhibitor EDTA and PMSF were faint than control lane. Crude protease of CSH5 (Figure 3.1 D), also showed inhibition with EDTA and PMSF. Bands were slightly faint compared to control. Crude protease of CSH6 (Figure 3.1 E), showed inhibition with EDTA only. A faint band was seen in lane with EDTA, and lanes with PMSF and Pepstatin A were of same intensity as of control. Crude protease of CSH7 (Figure 3.1 F), showed inhibition with PMSF and Pepstatin A. The intensity of bands were faint compared to control lane. It did not show any inhibition with EDTA. Crude protease of CSH8 (Figure 3.2

A), showed inhibition mainly with EDTA. It also showed inhibition with PMSF. A lane with PMSF was slightly faint compared to control lane. Also lane with Pepstatin A was very slightly faint. Crude protease of CSH 11 (Figure 3.2 D), showed slightly faint band in lane with PMSF and EDTA but not in Lane with Pepstatin A which indicates that it was slightly inhibited by PMSF and EDTA. Crude protease of Cc50 (Figure 3.3 A), showed very slight inhibition with PMSF as the intensity was very slightly faint compared to control and no inhibition was detected with EDTA and Pepstatin A at all.

Crude protease of FDSC isolates, Cc26, Cc119 and CSH4 was not strongly inhibited by any of the inhibitors. In zymogram of crude protease of Cc26 (Figure 3.4 A), bands of lanes with PMSF and Pepstatin A were very slightly faint compared to control lane. In zymogram of crude protease of Cc119 (Figure 3.4 B), bands of lane with PMSF were very slightly faint in intensity compared to control lane. In zymogram of crude protease of CSH4 (Figure 3.4 C), the inhibition of upper most band was seen in lanes with all 3 inhibitors but the lower bands were prominent in all lanes with same intensity which also indicate very slight inhibition.

Crude protease of *F. sacchari* isolates did not show strong inhibition in presence of any inhibitors. However, crude protease of Cc52 (Figure 3.5 A), Cc61 (Figure 3.5 B) and Cc215 (Figure 3.5 D) were slightly inhibited by PMSF. Crude protease of Cc167 ((Figure 3.5 C) was inhibited with PMSF and intensity of band in presence of inhibitor was half compared to control lane.

Crude protease of *F. incarnatum-equiseti* Cc172 was also not inhibited by any of the inhibitor (Figure3.6).

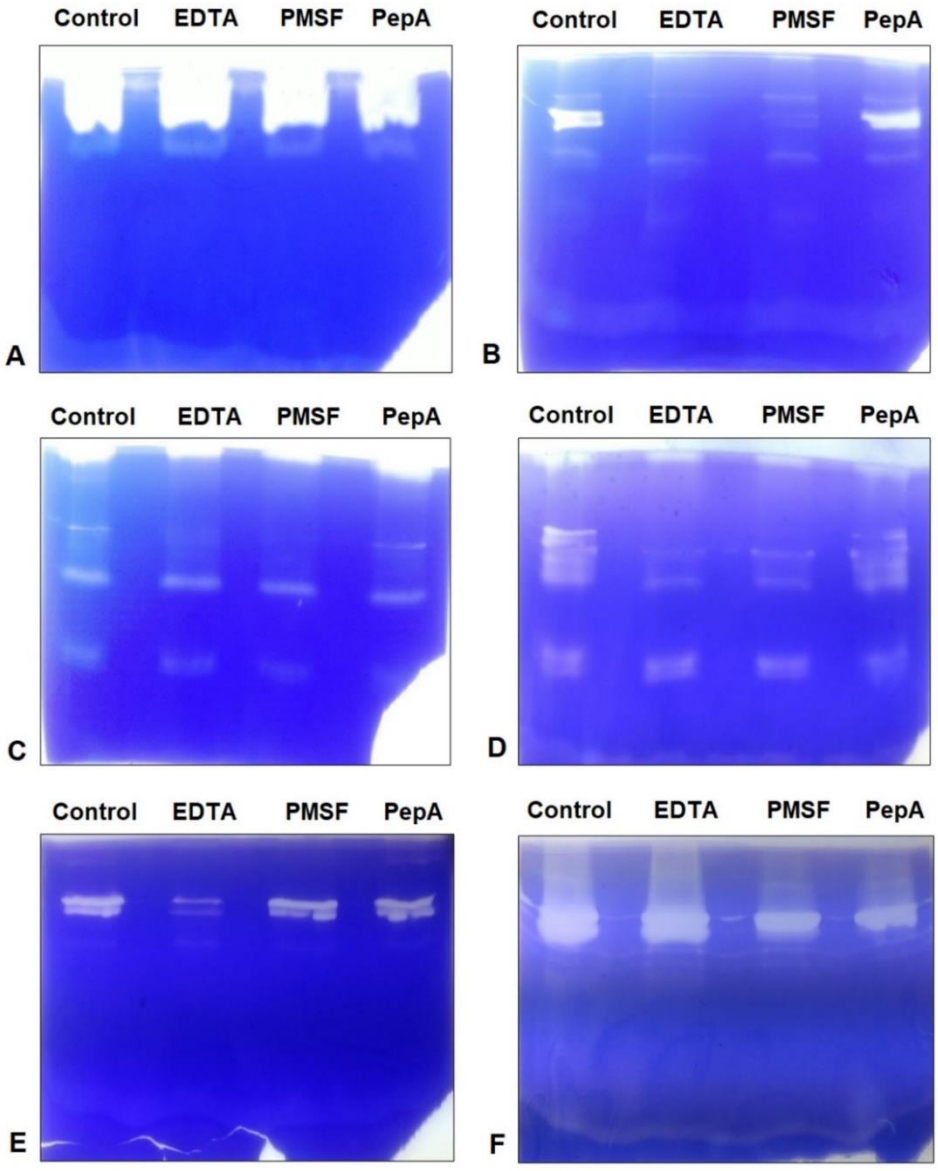

Figure 3.1: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of FSSC isolates. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM). (**A)** FSSC CSH1, **(B)** FSSC CSH2, **(C)** FSSC CSH3, **(D)** FSSC CSH5, **(E)** FSSC CSH6 and **(F)** FSSC CSH7.

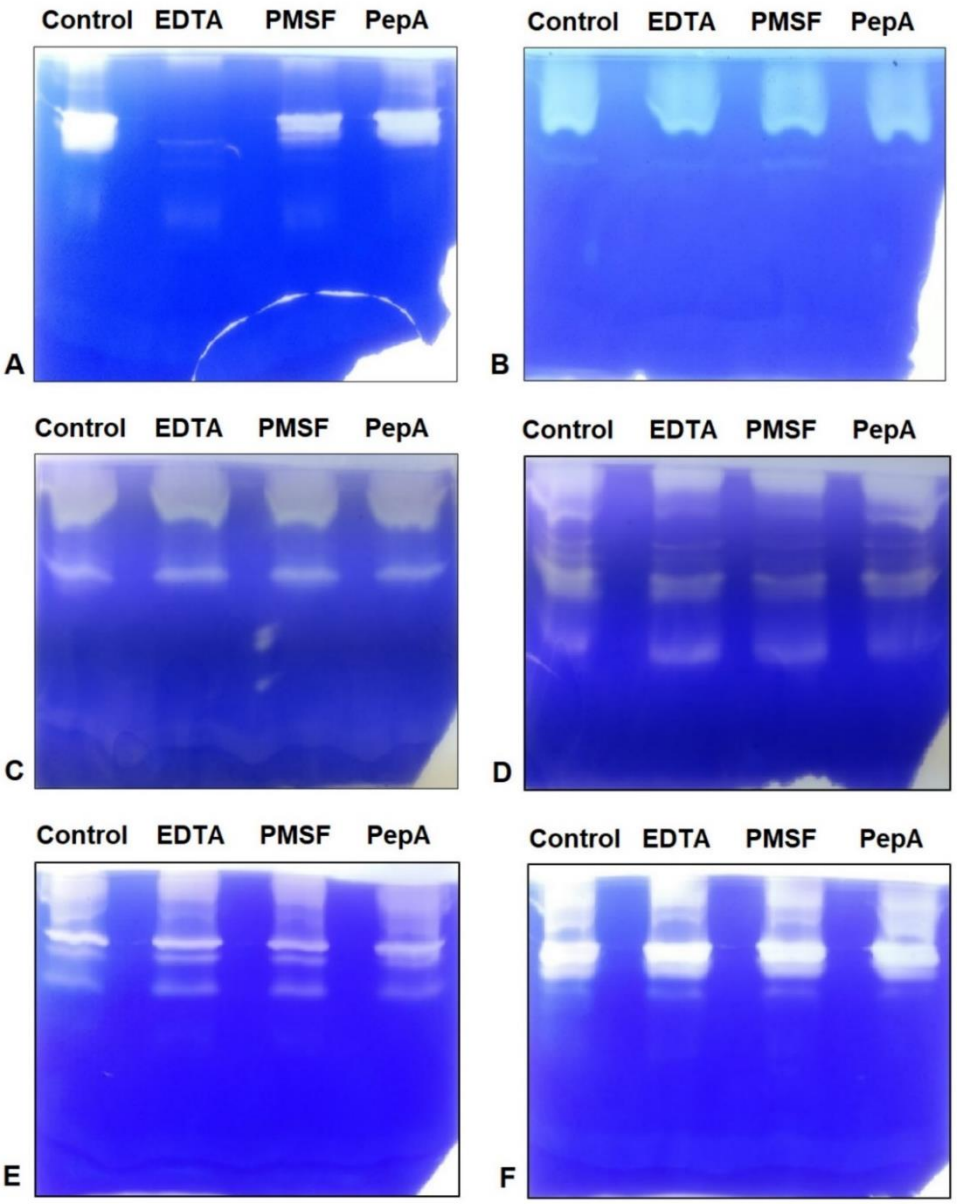

Figure 3.2: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of FSSC isolates. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM). (**A)** FSSC CSH8, **(B)** FSSC CSH9, **(C)** FSSC CSH10, **(D)** FSSC CSH11, **(E)** FSSC Cs1 and **(F)** FSSC Cs2.

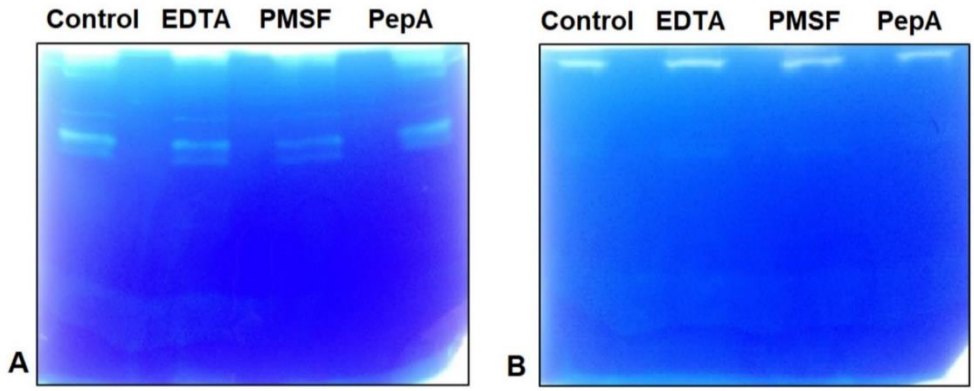

Figure 3.3: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of FSSC isolates. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM). (**A)** FSSC Cc50 and **(B)** FSSC Cc240.

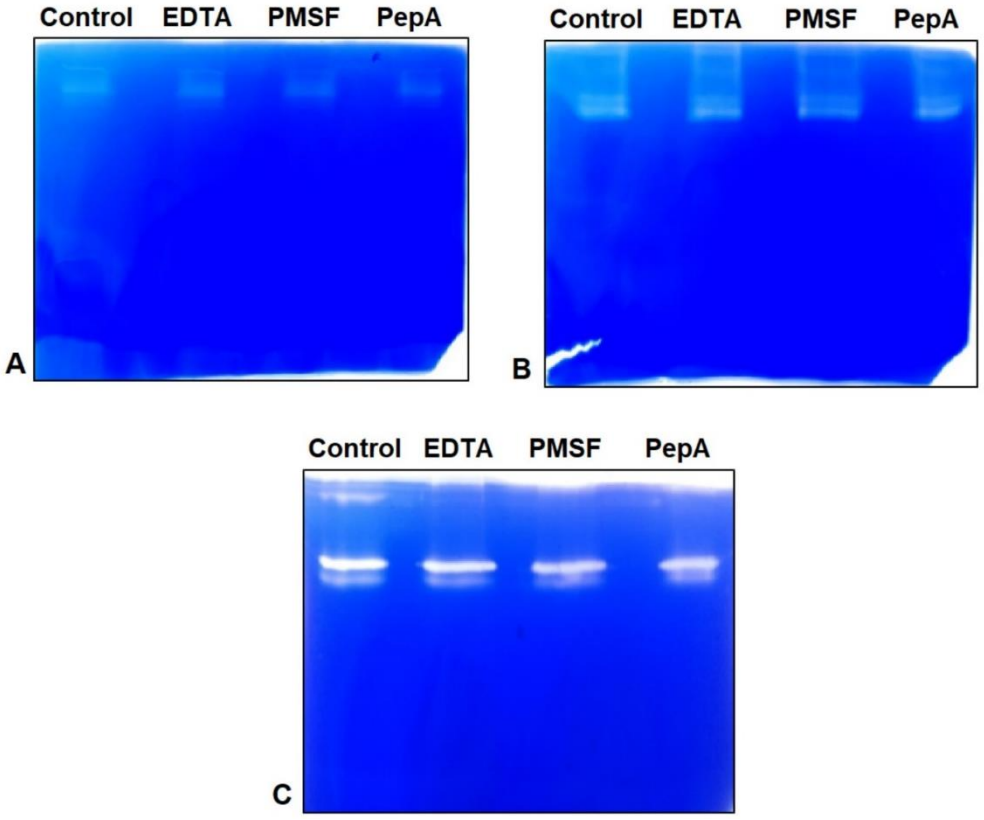

Figure 3.4: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of FDSC isolates. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM). (**A)** FDSC Cc26, **(B)** FDSC Cc119 and **(C)** FSSC CSH4.

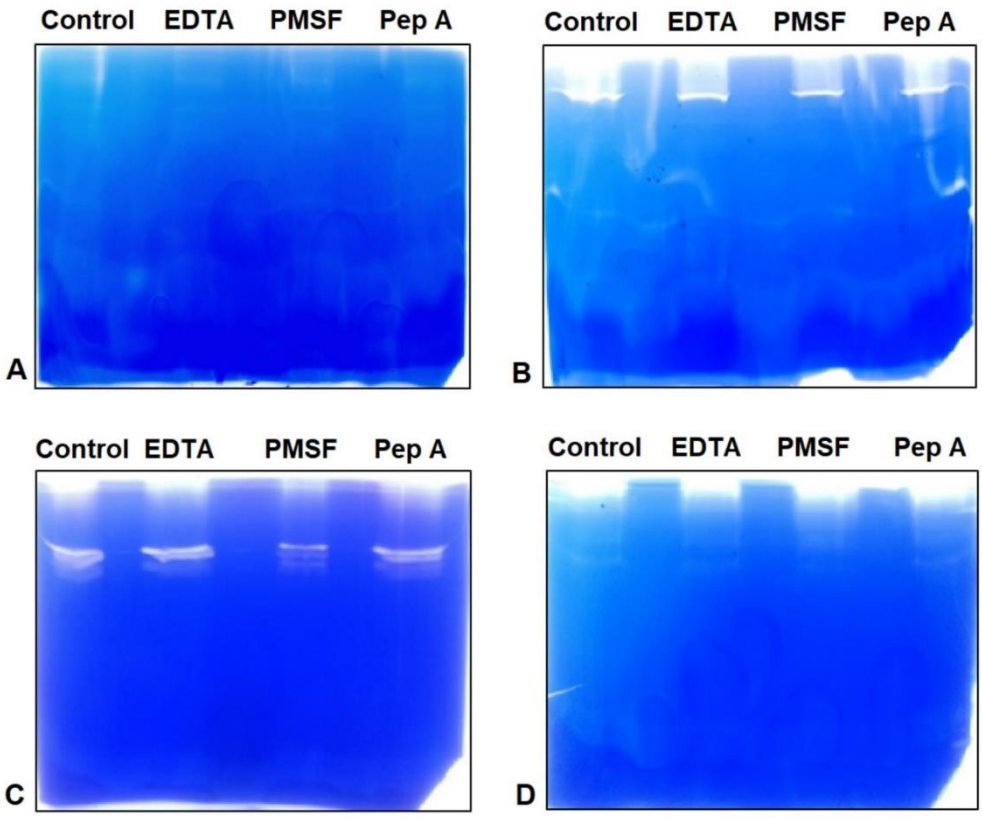

Figure 3.5: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of *F. sacchari* isolates. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM). (**A)** *F. sacchari* Cc52, **(B)** *F. sacchari* Cc61, **(C)** *F. sacchari* Cc167 and **(D)** *F. sacchari* Cc215.

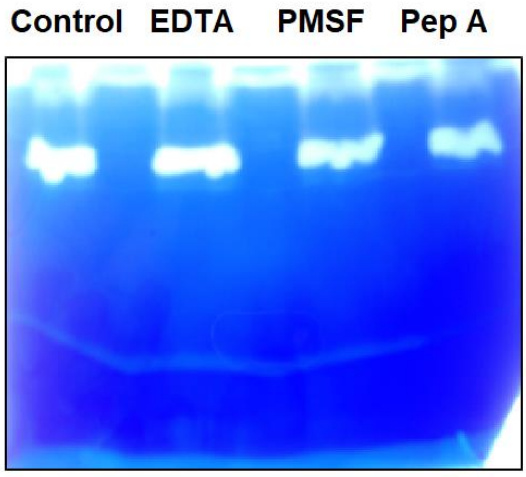

Figure 3.6: 12% SDS-PAGE gelatine (0.1%) zymogram of crude extracted protease of *F. incarnatum-equiseti* Cc172. Inhibitors used were EDTA (10.0mM), PMSF (5.0mM) and Pepstatin A (10.0µM).

3.3.3 Quantitative RT-PCR (qRT-PCR):

Quantitative RT-PCR was carried out for FSSC isolates as the whole genome sequence database is available for *N. haematococca*. UniProt KB was used to find out proteases present in *N. haematococca*. Proteases ranging from 50-100 kDa were selected. The gene sequence for these proteases was obtained from Ensembl Fungi and National centre for Bioinformatics (NCBI). TEF1 alpha was used as housekeeping gene. Among all FSSC isolates, expression of seven genes C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 was detected out of 14 selected genes using qRT-PCR. Maximum relative gene expression was found in genes C7Z7U2, C7YY94 and C7Z6W1. The relative gene expression of C7Z7U2 was maximum in CSH11 and lowest in Cc50. The expression of C7YY94 was highest in CSH11 and lowest in Cs1. The expression of C7Z6W1 was maximum in Cs1 and lowest in Cc50. The expression of genes C7ZNV5, C7YQJ2, C7YVF3 and C7Z436 was low compared to other genes. However, expression of genes C7Z7NV5, C7YQJ2, C7YVF3 and C7Z436 was highest in Cs1 compared to other isolates. Relative gene expression of all FSSC isolates is represented in Figure 3.7, Figure 3.8 and Figure 3.9.

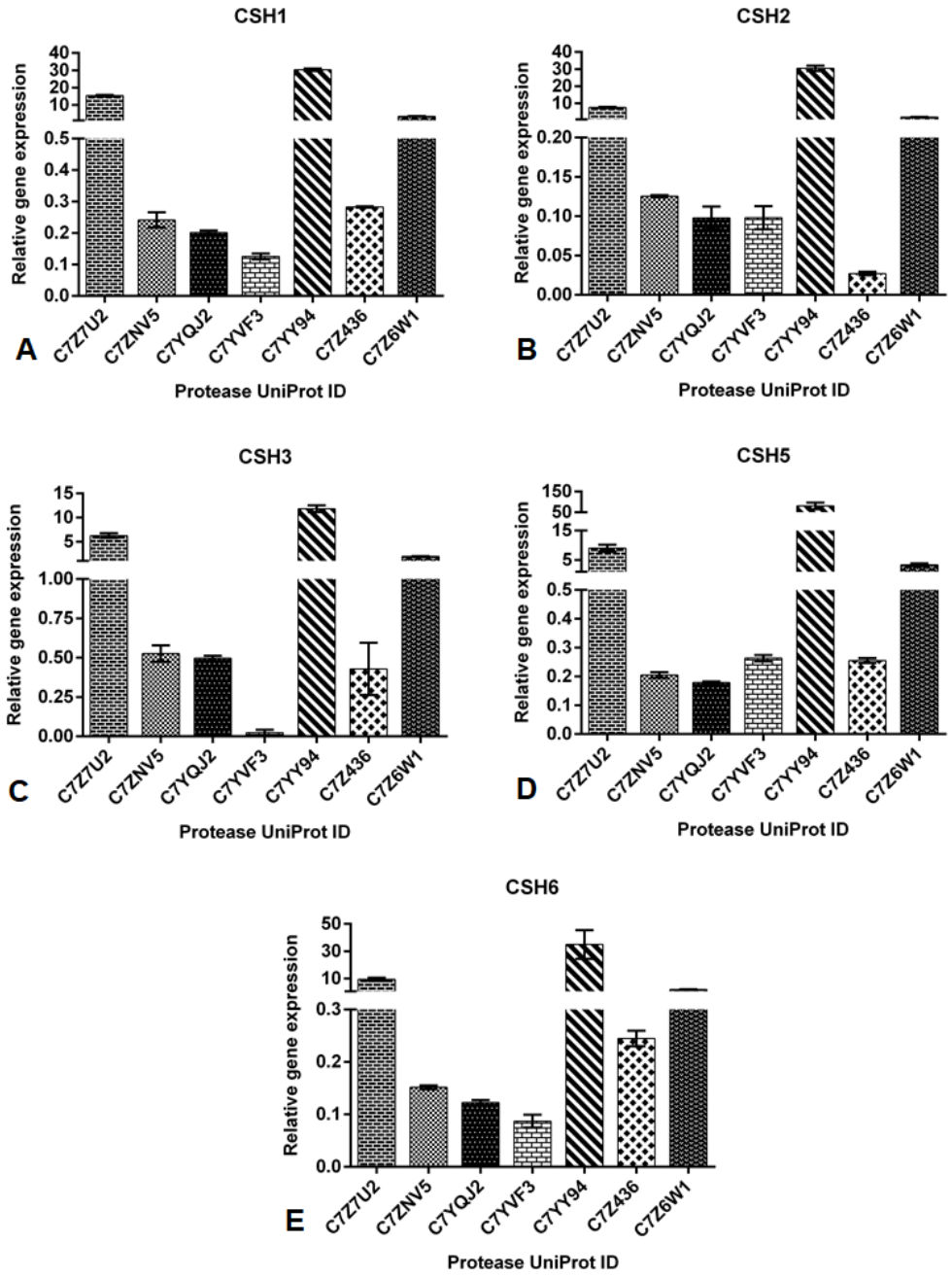

Figure 3.7: Relative gene expression of C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 in FSSC isolates. (**A)** FSSC CSH1, **(B)** FSSC CSH2, **(C)** FSSC CSH3, **(D)** FSSC CSH5 and **(E)** FSSC CSH6. Expression of C7Z7U2, C7YY94 and C7Z6W1 was highest and statistically significant (p<0.05).

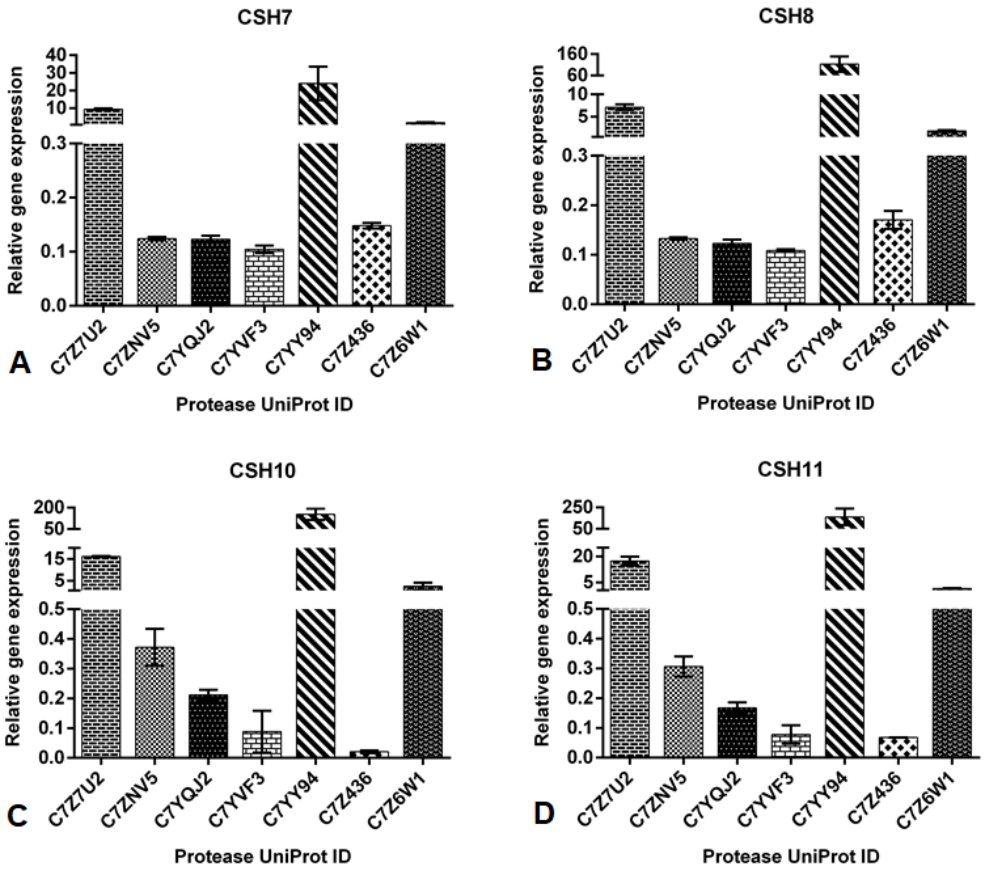

Figure 3.8: Relative gene expression of C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 in FSSC isolates. (**A)** FSSC CSH7, **(B)** FSSC CSH8, **(C)** FSSC CSH10 and **(D)** FSSC CSH11. Expression of C7Z7U2, C7YY94 and C7Z6W1 was highest and statistically significant (p<0.05).

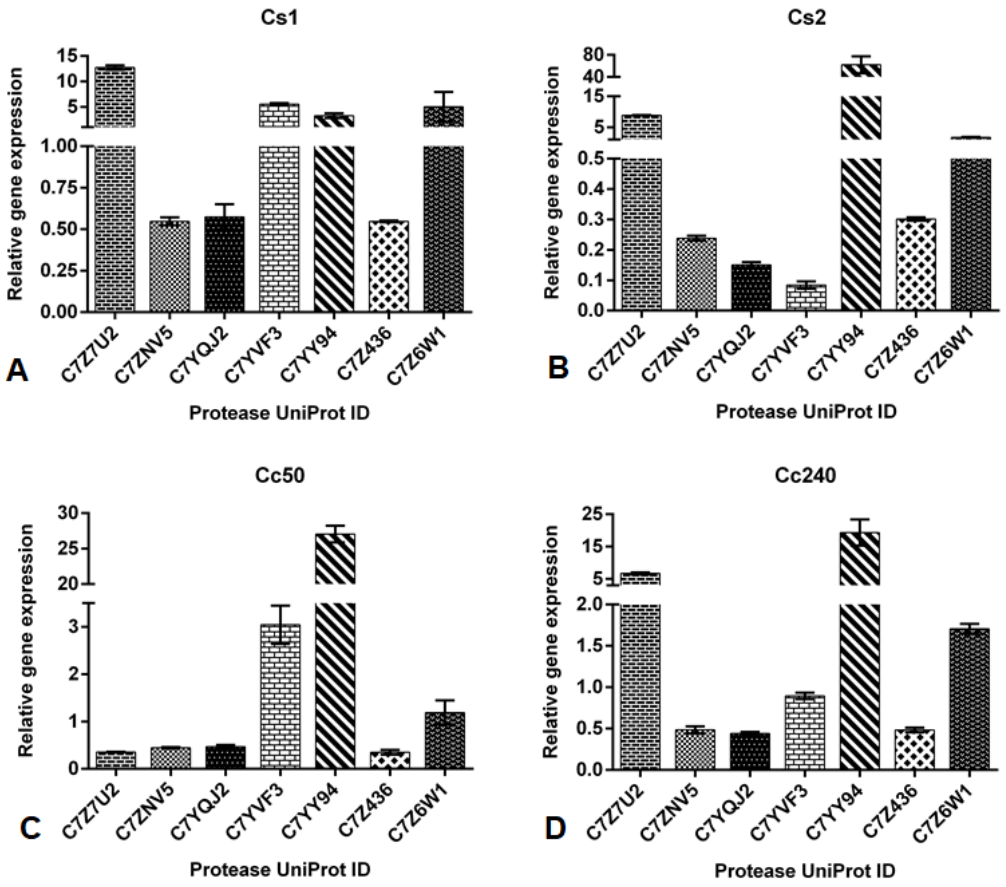

Figure 3.9: Relative gene expression of C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 in FSSC isolates. (**A)** FSSC Cs1, **(B)** FSSC Cs2, **(C)** FSSC Cc50 and **(D)** FSSC Cc240. Expression of C7Z7U2, C7YY94 and C7Z6W1 was highest and statistically significant (p<0.05) in Cs1, Cs2 and Cc240. Expression of C7YVF3 and C7YY94 was highest and statistically significant (p<0.05) in Cc50.

3.3.4 Purification of protease:

Crude acetone precipitated extract of Cs1 was subjected to purification by column chromatography. Casein agarose was used for the separation of the proteins from the crude sample. Affinity chromatography works on the principle that different proteins have different affinity for the matrix. Hence, every protein will bind with different affinity to the matrix. So, proteins with low affinity will be eluted first and proteins with higher affinity will be eluted in the last. 1 ml fractions were collected from column purification and each fraction was subjected for azocasein assay to estimate specific activity and Bradford assay to estimate protein concentration.

Figure 3.10 shows specific activity and protein concentrations of collected fractions. It can be seen that fraction 14 and 15 were having highest specific activity. Fractions near fraction 15 were subjected to 12% SDS-PAGE followed by silver staining. After silver staining of fractions no. 13, 14, 15, 16 and 17, single intense band in fraction no. 15 was seen (Figure 3.11). There was also a single less intense band in fraction 14. The position of the band seen in fractions 14 and 15 was responsible for giving high protease activity in the azocasein assay compared to set of bands observed in fractions 13, 16 and 17. Thus, fraction 15 was further subjected to characterization by mass spectroscopy.

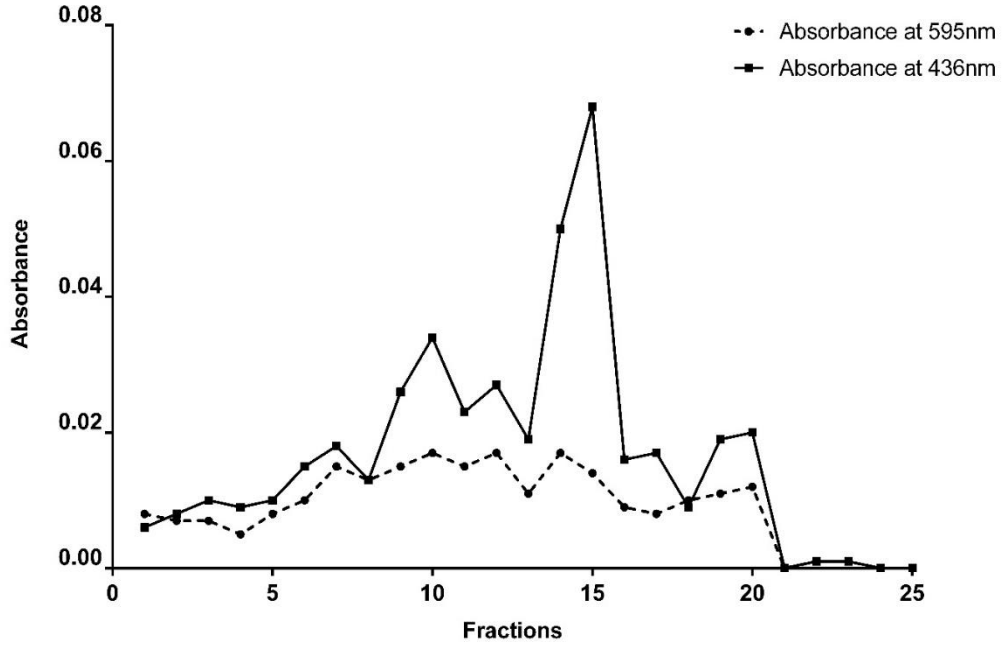

Figure 3.10: Purification of protease using casein agarose affinity column chromatography. Black dotted line represent the total protein content and black continuous line represent the activity of the protein. Maximum activity was seen in fraction 15.

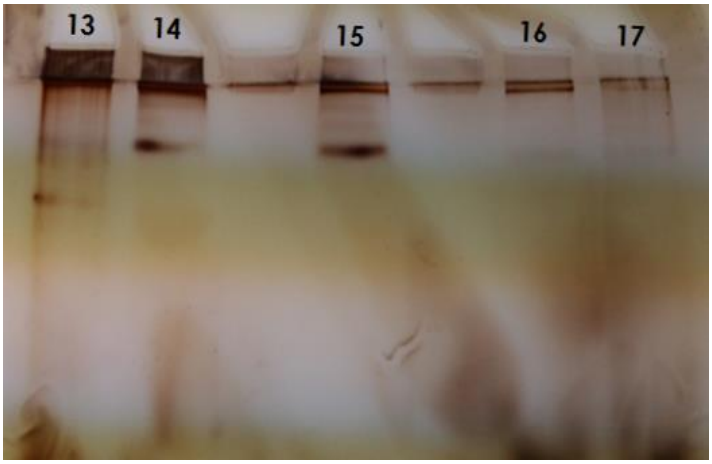

Figure 3.11: Silver stained 12% SDS-PAGE of purified fraction 13, 14, 15, 16 and 17. Fraction 14 and 15 gave presence of intense single band. Highest protein concentration was seen in fraction 15.

3.3.5 Identification of protease using LCMS-MS (Liquid chromatography-Mass spectroscopy):

Acetone precipitated crude enzyme extract and purified 15th fraction were used for protein identification. The crude enzyme extract was passed through 10kDa M.W. Cut-off column to remove small and degraded peptides as wells amino acids. The protein concentration was estimated using Bradford assay and subjected to HR-LC-MS/MS. After purification from column, fraction 15 showed maximum specific activity and in SDS-PAGE, fraction 15 gave a single intense band. This fraction no. 15 was also used for protein identification. Table 3.4 shows proteases identified from exoproteome of concentrated crude extract of Cs1 and from column purified fraction 15. Many of the identified proteins were not having signal sequence. They are secreted by non-classical pathway. The obtained sequences were identified using proteome discoverer 2.0 with in-built sequenceHT and Masscott software. Also the sequences were blasted for peptide match using BLASTP from NCBI and protein blast from UniProtKB.

In exoproteome analysis, the peptide sequences of Cs1 was matching with number of proteins from different species but the hits with proteases were only six. The mass spectrometry was repeated three times. Following are the proteases detected in exoproteome when blasted on NCBI and UniProtKB. 1. Mitochondrial intermediate peptidase (*C. albicans*), 2. Pro-apoptotic serine

protease (*S. cereviseae*), 3. Carboxypeptidase (*N. haematococca*), 4. Aminopeptidase (*Pseudomonas*), 5. Metallopeptidase (*Pseudomonas*), 6. Predicted protein (having peptidase activity) (*N. haematococca*). The column purified fraction shows the similarity with tripeptidyl amino peptidase**.** The respective accession number, calculated isoeclectric point, molecular weight is mentioned in Table 3.4.

Table 3.4: Protease identified from FSSC isolate Cs1 by HRLC-MS/MS (orbitrap) in exoproteome and after column purification.

No.	Function	pI	M.W. (kDa)	Nearest informative homologue given by BlastP Accession [organism]	Nearest informative homologue given by UniprotKB Accession [organism]
Proteases identified in exoproteome					
1	Mitochondrial intermediate peptidase	6.44	89.3	3646074 (*Candida albicans*)	Q59RK9 (*C. albicans*)
2	Pro-apoptotic serine protease	5.96	110.8	125863594 (*Saccharomyces cerevisiae*)	A6ZRW1 (*S. cerevisiae*)
3	Carboxypeptidase	5.63	62.1	9675077 (*Nectria haematococca*)	C7YVF3 (*N. haematococca*)
4	Probable cytosol aminopeptidase	7.66	52.4	229359445 (*Pseudomonas fluorescens*)	C3K6G5 (*P. fluorescens*)
5	Metallopeptidase AprA	4.88	49.4	757867863 (*P. panacis*)	A0A0C5CJR8 (*P. panacis*)
6	Predicted protein	5.53	58.7	9677116 (*N. haematococca*)	C7YVP3 (*N. haematococca*)
Protease identified in fraction 15 after purification by casein agarose column chromatography					
7	Tripeptidyl aminopeptidase	5.23	58.0	927755098 (*Fusarium langsethiae*)	A0A0M9ESD1 (*F. langsethiae*)

3.3.6 Development of explant goat cornea culture:

After collecting the eye ball from abbretorier's, the corneas were sterilised with povidone iodine, gentamycin, streptomycin and penicillin. The dissected explants of cornea were placed in a sterile 12 well tissue culture plates contained artificial corneal button and DMEM medium for a period of 15 days (Figure 3.12) and viability of intact cornea and epithelial cells was measured at various time intervals. The viability of corneal epithelial cells was assessed using the trypan blue assay and the viability of the entire cornea was assessed using the MTT assay. Both the assays were carried out at alternate day till 20 days. Morphologically corneas were also observed for opacity.

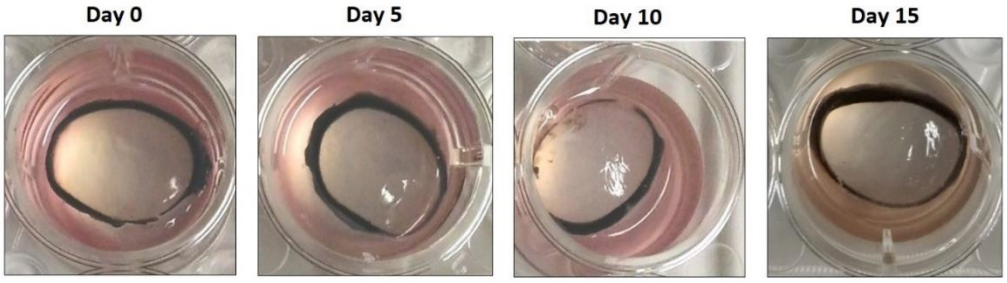

Figure 3.12: *Ex vivo* caprine cornea maintained in Dulbecco's modified eagle's medium (DMEM) at days 0, 5, 10, and 15. Cornea was transparent up to 15th day.

3.3.7 MTT assay:

The yellow tetrazolium MTT is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

The intact cornea was used for measuring the overall viability using the MTT assay. The cells of cornea were metabolically active and were able to reduce MTT to formazon a purple colour complex. The solubilised purple formazon complex was spectrophotometrically measured at 570nm with a reference wavelength higher than 650 nm. Specific absorbance was calculated as follows. Specific absorbance = Absorbance (570nm) (test) – Absorbance (570nm) (blank) – Absorbance (>650nm) (test). Figure 3.13 shows the purple

colour complex taken up by the MTT treated cornea which indicates the viability of cornea. The readings at 570nm were taken at day 5, day10 and day 15. Figure 3.14 shows the graph of specific absorbance at 570 nm at different days. The specific absorbance was 0.038±0.00, 0.176±0.00, 0.210±0.01 and 0.203±0.00 at day 0, day 5, day 10 and day 15, respectively. After 15th day the cornea's opacity was increased and specific absorbance decreased. The maximum viability was seen at day 10.

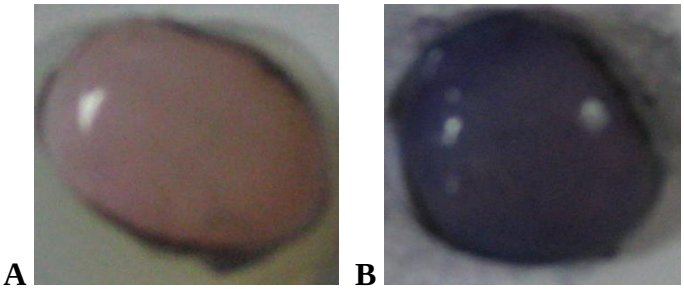

Figure 3.13: Cornea before and after MTT induction. (A) Cornea without MTT induction and **(B)** Purple formazone complex taken up by cornea after MTT induction.

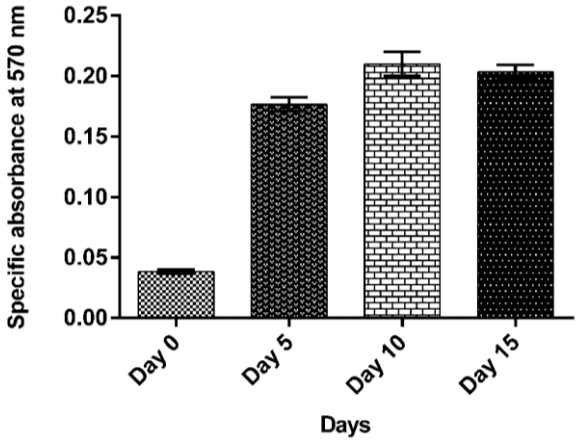

Figure 3.14: Specific absorbance of formazon complex at 570 nm at Day 0, day 5, day 10 and day 15 using MTT assay to assess the viability of cornea.

3.3.8 Trypan Blue Assay:

To carry out trypan blue assay, the epithelial cells were separated from cornea by trypsin treatment. The collected cells were pelleted down. Figure 3.15 shows the separated epithelial cells. To these collected cells, trypan blue solution was added and % viable cells were counted using haemocytometer. The cell count was carried out at different time interval. Figure 3.16 shows the

graph of % viable cells at day 0, day5, day 10 and Day 15. The % viable cells were 96.266±0.11, 95.80±0.02, 94.210±0.02 and 88.280±0.02 at day 0, day 5, day 10 and day 15, respectively. The cell count was decreased from day 15. As the corneas were cultured they become swollen. Up to 10th day cornea were completely intact but at 15th day the cornea loses its intactness and therefore we did not culture cornea beyond 15 days. The cornea was completely transparent up to 10th day the transparency was reduced at 15th day however cornea did not become opaque up to 15th day.

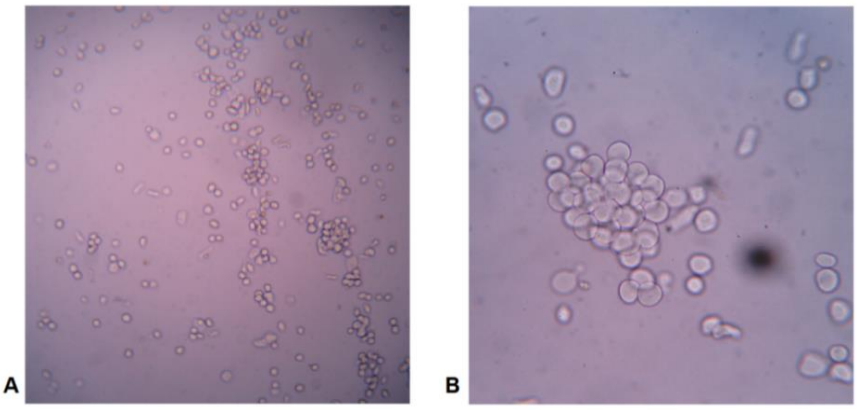

Figure 3.15: Corneal epithelial cells after trypsin treatment. (A) 10x magnification and **(B)** 40X magnification

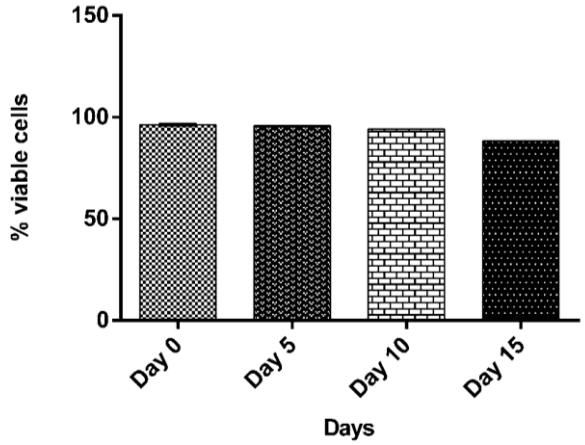

Figure 3.16: The graph of % viable cells at Day 0, day 5, day10 and day 15 using trypan blue assay. Viability was up to 88% on 15th day.

3.3.9 Infection of cornea with *Fusarium* isolate Cc61:

To carry out infection, the corneas after sterilization procedure were scratched from surface to make small wound for development of infection. To cause infection, the pathogen should be present on to the cornea and it should be viable and culturable. The culture was grown freshly for infection. The fungal

isolate initially used for development and standardization purpose was Cc61. The fungal isolate was grown on PDA and at 8th day the spores were collected and counted using haemocytometer. The corneas were infected with an inoculum size of 932±46 spores. To check the viability of spores after infection the spores were collected from top of infected cornea using sterile microbial loop and streaked on PDA and NA plates to check for fungal growth and bacterial contamination, respectively. Also the spent media was streaked every time when media was changed to check for fungal growth and bacterial contamination.

After infection, the corneas were observed for infection progress every day up to day 10th. Figure 3.17 shows the infected cornea with progression of infection at different days. Changes in the morphology and transparency of the cornea with respect to the control could be seen. Up to day 8, the opacity of the cornea increased. To check the progression of infection inside the cornea, the corneas were harvested at day 2, day 4 and day 8. Corneas were completely degraded by day 10. The harvested corneas were proceeded for keratome sectioning followed by H-E staining. Figure 3.18 shows the histology of infected corneas at different time interval. At the 2nd day, the epithelium was completely disrupted and the fungi started penetrating in the upper stroma. At the 4th day, the fungi were more concentrated at the upper half of the cornea and presence of long hyphae compared to second day. At 8th day, half of the stroma was completely disrupted and lower half also contained fungal hyphae. Also the morphology of infected corneal stroma was changed with progression of infection. At 10th day the histological processing of cornea was not possible because the entire cornea was degraded.

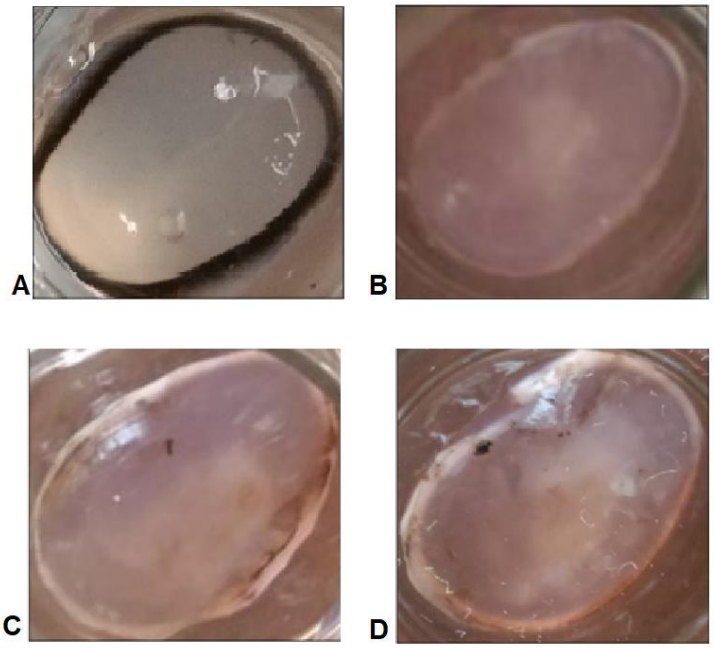

Figure 3.17: *Ex vivo* cornea infected with Cc61 at day 2, day 4 and day 8. Infected corneas show haze and fungal growth compared to uninfected cornea.

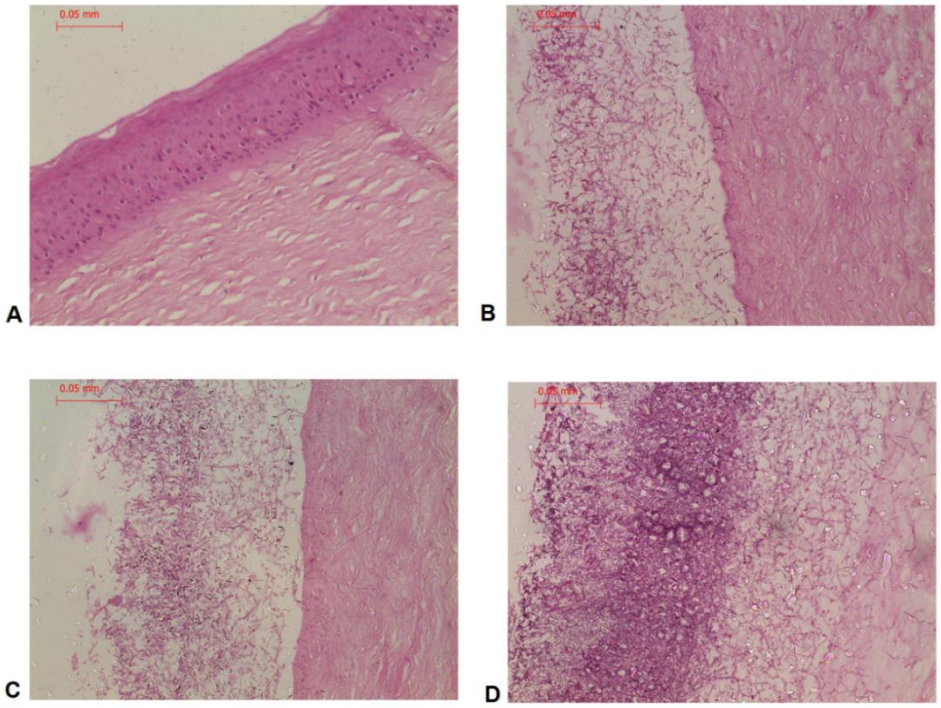

Figure 3.18: Histology of uninfected and infected *ex vivo* corneas. Uninfected cornea show the complete epithelial layer. Infected corneas show fungal growth and penetration of hyphae in to stroma and degraded epithelial layer and uninfected cornea. **(A)** Uninfected, **(B)** Day 2, **(C)** Day 4 and **(D)** Day 8.

3.3.10 Quantitative Real time PCR (qRT-PCR):

Expression studies were carried out from corneas harvested after infection on day 2, day 4 and day 8. Five proteases were selected on the basis of molecular weight and their extracellular property (signal peptide analysis). Figure 3.19 shows the relative gene expression of proteases; C7Z0E6, C7ZFW9, C7Z7U2, C7ZNV5, and C7YY94; of which C7Z0E6, C7Z7U2, C7ZNV5 show more expression among all. The difference between expression of C7Z0E6 at 4th and 8th day is statistically significant, but not at 2nd and 4th day. C7Z7U2 was up-regulated at 2$^{nd\ day}$, 4th day and 8th days and attained statistical significance. For C7ZNV5, the up-regulation was statistically significant at 2nd day and 4th day but not at 8th day.

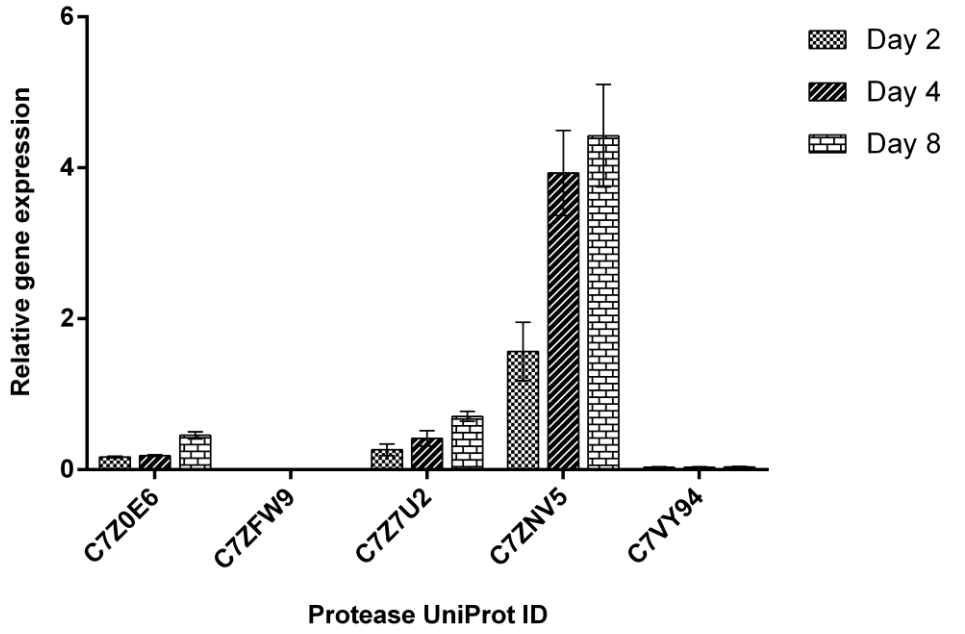

Figure 3.19: Relative gene expression of the C7Z0E6, C7ZFW9, C7Z7U2, C7ZNV5, and C7YY94 in Cc61. C7ZNV5 expression was highest at 2nd, 4th and 8th day. Statistically significant difference was considered at p>0.05.

3.3.11 Infection of cornea with FSSC Cs1, CSH3 and Cc50:

After standardization of an ex-vivo infection model as well as expression studies in one isolate 3 isolates were selected with highest, lowest and intermittent specific activity which were Cs1, Cc50 and CSH3, respectively based on an *in-vitro* specific activity and expression studies. After standardization of an *ex vivo* infection model, an *in vitro* expression studies

was carried out on more 9 protease genes along with the other 5 genes with M.W. range from 50-100 KDa in all FSSC isolates.

Here, some modifications were done to develop infection model which were mentioned in Materials and method. Infection load used was 10^4 spores. Media was changed after every 6 hours. Figure 3.20, Figure 3.21 and Figure 3.22 shows the infected cornea with progression of infection at different days in isolate Cc50, CSH3 and Cs1, respectively. The growth of fungus increased gradually. The streak from medium was negative on NA plates which indicated no bacterial contamination. If any of the eyes got contaminated, the whole experiment was repeated again with new set of corneas. The progression of infection was rapid in case of Cs1 compared to CSH3 and Cc50. On 2^{nd} day, the fungal growth was more in Cs1 compared to CSH3 and Cs1 but compared to Cc50, the growth was slow in CSH3 on 2^{nd} day. On 4^{th} day, the growth was more than 2^{nd} day and in Cs1 and CSH3, the fungal growth was similar and in Cc50, the growth was less on 4^{th} day. On 8^{th} day, growth was maximum and covered whole cornea in all 3 infection model of Cs1, CSH3 and Cc50.Compared to CSH3 and Cc50, the fungal growth was more in Cs1 on 8^{th} day and limbal region was also not visible. In Cc50, the fungus has covered the cornea but limbal region was visible and fungal mat was also less compared to Cs1 and CSH3. Figure 3.23 shows the corneal histology at 8^{th} day of Cc50, CSH3 and Cs1. During initial experiment, the fungal growth and cornel histology showed the maximum growth and penetration on day 8 so in the infection model with Cs1, CSH3 and Cc50, keratome sectioning followed by H-E staining was carried out Day 8. In Cc50, the epithelial layer was partially degraded and penetration of fungus into the stroma was very less (Figure 3.23B). In CSH3, the epithelial layer was degraded and fungus penetrated in the upper half part of the stroma (Figure 3.23C). In Cs1, the epithelial layer was completely degraded and fungal hyphae penetrated deep into the stroma (Figure 3.23D).

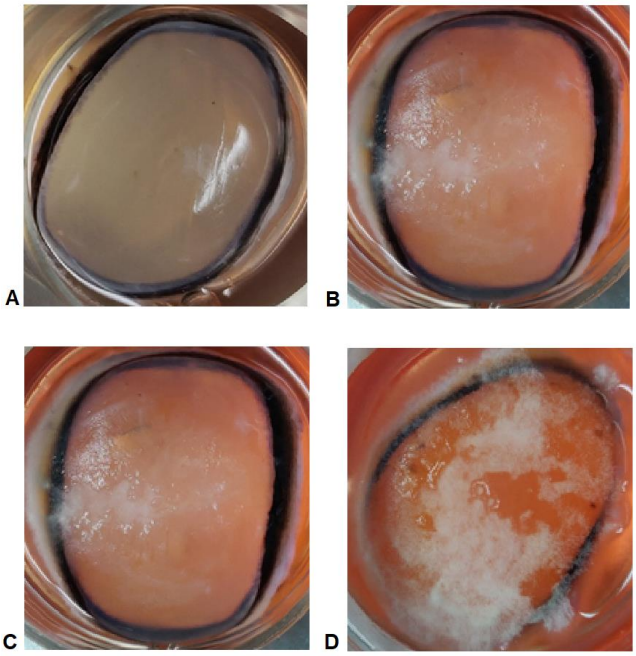

Figure 3.20: *Ex vivo* cornea infected with Cc50 at day 2, day 4 and day 8. Infected corneas show haze and fungal growth compared to uninfected cornea.

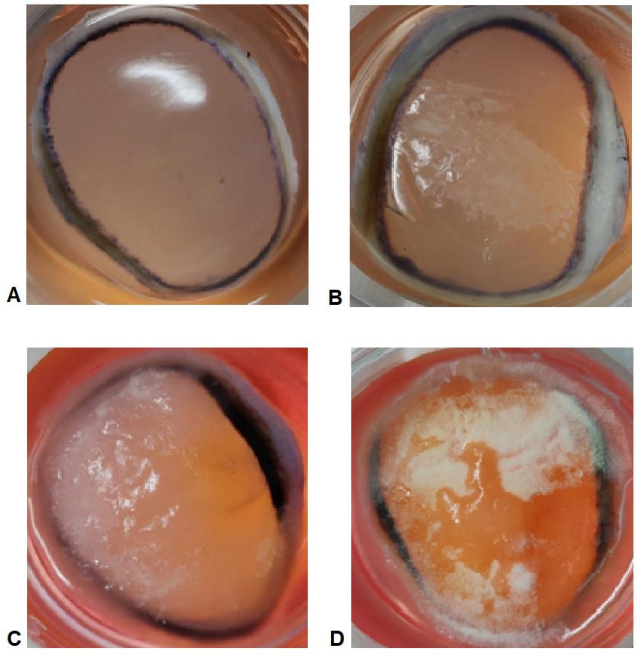

Figure 3.21: *Ex vivo* cornea infected with CSH3 at day 2, day 4 and day 8. Infected corneas show haze and fungal growth compared to uninfected cornea.

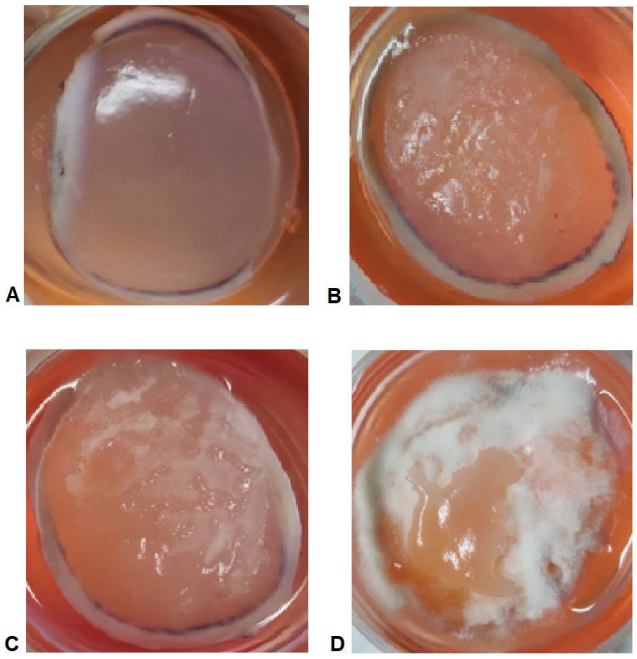

Figure 3.22: *Ex vivo* cornea infected with Cs1 at day 2, day 4 and day 8. Infected corneas show haze and fungal growth compared to uninfected cornea.

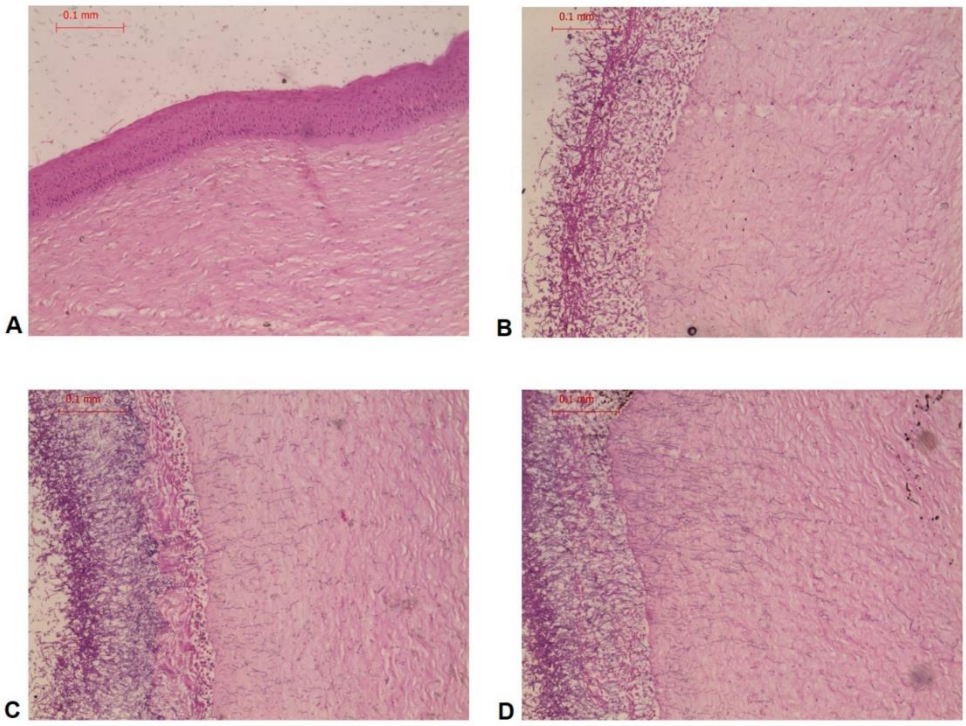

Figure 3.23: Histology of control and infected *Ex-vivo* corneas. Uninfected cornea show the complete epithelial layer. Infected corneas show fungal growth and penetration of hyphae in to stroma and degraded epithelial layer compared to uninfected cornea. (**A)** Uninfected (control) cornea, **(B)** Cornea infected with Cc50, **(C)** Cornea infected with CSH3 and **(D)** Cornea infected with Cs1.

3.3.12 Specific activity estimation during an *Ex vivo* infection condition:

The spent medium was used to estimate protease activity using azocasein assay and protein estimation by using Bradford's assay. Figure 3.24 shows the specific activity of protease at day 2, day 4, day 6, day 8 and day 10 in Cc50, CSH3 and Cs1. In Cc50, the specific activity at day 2, day 4, day 6, day 8 and day 10 was 2.765±0.21 units/mg, 3.117±0.06 units/mg, 6.322±0.53 units/mg, 14.348±0.39 units/mg and 3.074±0.01 units/mg, respectively. In CSH3, the specific activity at day 2, day 4, day 6, day 8 and day 10 was 4.596±0.30 units/mg, 9.986±0.74 units/mg, 10.273±1.21 units/mg, 18.862±1.32 units/mg and 11.333±2.34 units/mg, respectively. In Cs1, the specific activity at day 2, day 4, day 6, day 8 and day 10 was 2.383±0.19 units/mg, 5.545±0.73 units/mg, 10.621±3.75 units/mg, 25.050±2.42 units/mg and 6.495±2.13 units/mg, respectively. The specific activity in all isolates was highest at 8th day. Maximum specific activity was observed in Cs1 followed by CSH3 and Cc50.

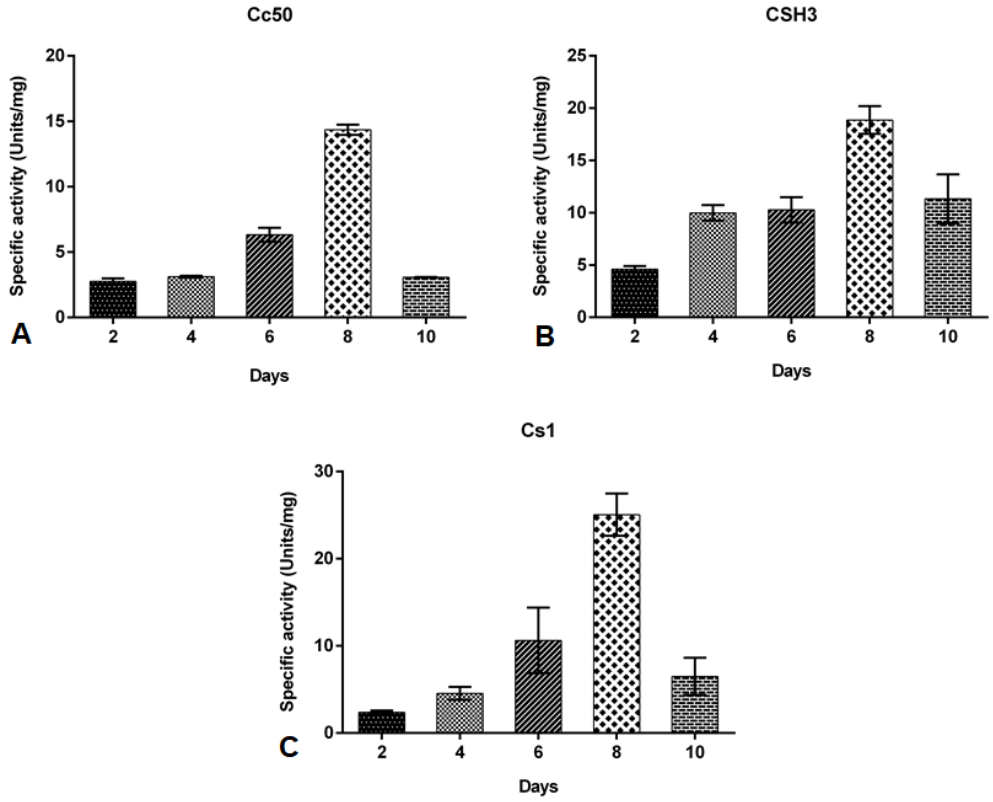

Figure 3.24: Specific activity estimation using azocasein assay during an *ex vivo* infection condition. (A) Cc50, **(B)** CSH3 and **(C)** Cs1. Statistically significant difference was considered at $p>0.05$.

3.3.13 Quantitative real time PCR (qRT-PCR):

Maximum specific activity was seen on day 8th in all 3 FSSC isolates during an *ex vivo* infection condition. Hence, the cornea harvested at 8th day was used for RNA isolation, cDNA synthesis and qRT-PCR. From the protease genes selected for study, expression was seen in C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1. MTCC 2935 was used as control strain. Figure 3.25 shows relative gene expression of C7Z7U2, C7Z7NV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 in Cc50, CSH3 and Cs1. In an *ex vivo* infection condition, among all seven expressed genes, C7Z6W1 showed highest expression followed by C7Z7U2 and C7YQJ2. Among all 3 isolates, the maximum gene expression was observed in Cs1 and minimum gene expression was seen in CSH3. In Cc50 (Figure 3.25A), higher expression was seen in C7Z6W1, C7Z7U2 and C7YQJ2. In CSH3 (Figure 3.25B), higher expression was seen in C7Z6W1, C7Z7U2, C7YQJ2, C7Z436 and C7YVF3. In Cs1 (Figure 3.25C), up-regulation was seen in C7Z6W1, C7YVF3, C7YQJ2, C7Z7U2, C7Z436 and C7ZNV5.

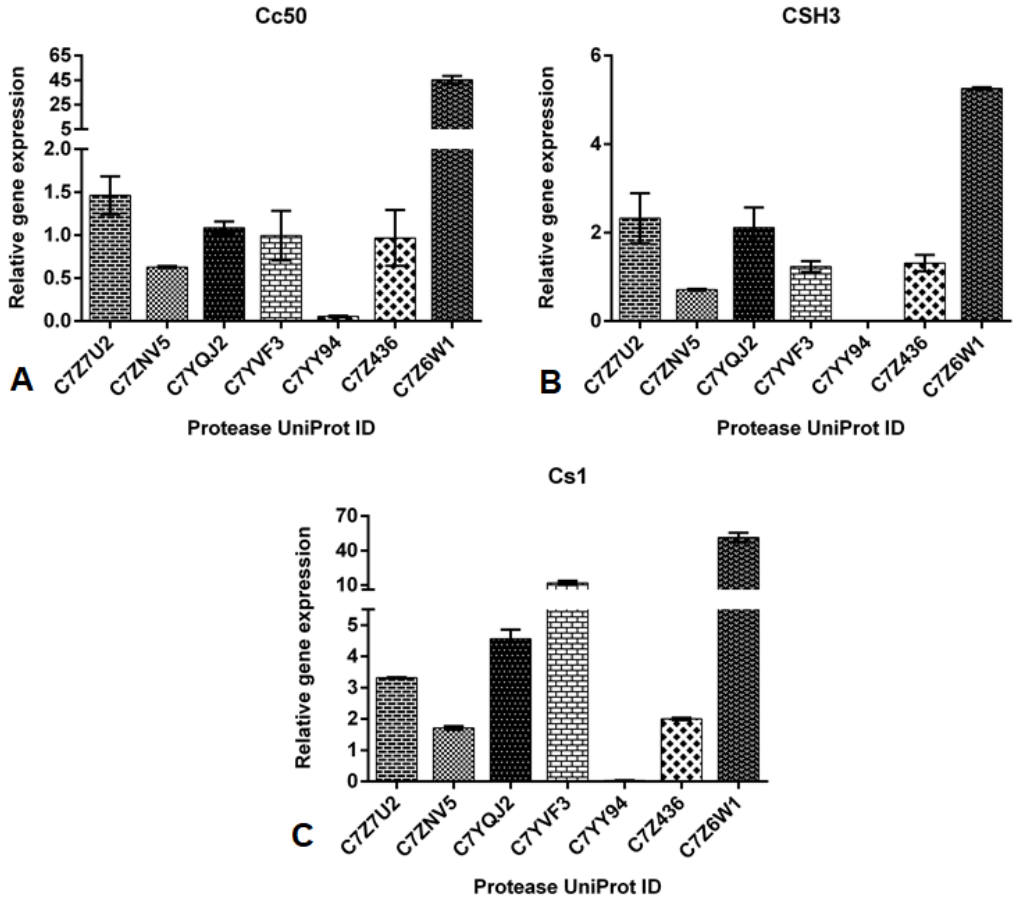

Figure 3.25: Relative gene expression of the C7Z0E6, C7ZFW9, C7Z7U2, C7ZNV5, and C7YY94 in an *ex vivo* infection condition. (A) FSSC Cc50, **(B)** FSSC CSH3 and **(C)** FSSC Cs1. C7Z6W1 expression was highest in all 3 FSSC isolates. Statistically significant difference was considered at $p>0.05$.

3.4 Discussion:

Proteases are secreted by pathogenic fungi during the infection. Proteases along with other secretory hydrolyting enzymes, leads to formation of macromolecules which are used by fungus for survival inside the host. (Dunaevsky *et al*., 2003). Secreted proteases belongs to several different families; aspartic protease, serine protease, cysteine protease, metalloprotease. Pathogenic fungi is also known to secrete aminopeptidase, carboxypeptidase and dipeptidyl peptidase. (Monod *et al*., 2002)

Host components like serum proteins, structural protein and antimicrobial peptides are potential targets for microbial proteases. Cleavage and degradation of these protenaceous compounds allows the pathogen to escape

and modulate host immune system and to gain nutrition. It also help them in adherence and dissemination in the host.(Armstrong, 2006; Santos, 2011).

Proteases are mainly produced and secreted highly by pathogenic fungi when it is present in protein rich environment as a sole source of nutrition such as nails, hair and cornea and hence becomes a very important virulence factor.

The present work includes, specific activity estimation, characterization and expression studies of proteases genes in an *in vitro* as well as in an *ex vivo* infection condition from keratitis causing *Fusarium* spp. Specific activity estimation, characterization of proteases and genes involved in their activity are well documented in *Aspergillus* spp., *Candida* spp., *Saccharomyces* spp. and other filamentous fungi (Monod *et al*., 2002; Santos, 2011). *Fusarium* spp. are well known to be plant pathogenic fungi and is an opportunistic pathogen, occasionally cause infection to animals and humans (Cutuli *et al*., 2015; Nucci and Anaissie, 2007; O'Donnell *et al*., 2016; Salter *et al*., 2012). There are increasing reports of human infection by *Fusarium*. There are several reports on protease characterization in *F. oxysporum*, *F. moliniforme*, *F. verticelloides* and *F. solani*, and on *F. delphenoides* reports are very few (Barata *et al*., 2002; Gajjar *et al*., 2013; Rodier *et al*., 1997; Rodríguez *et al*., 2017).

A 45 KDa metallopeptidase with optimum pH of 7.2 has been characterized from *F. moliniforme* (Rodier *et al*., 1997). Kolaczkowska and group had reported purification and characterization of 38 KDa aspartic protease from *F. moliniforme* culture filtrate with optimum pH of 3.2 (Kolaczkowska *et al*., 1983). Dudley and Chick, (1964) reported collagen bundles destruction by *F. moliniforme* in rabbit model of keratitis. A 41 KDa trypsin like alkaline serine proteases has been characterized in *F. oxysporum* with optimum pH of 8.0 (Barata *et al*., 2002). Trypsin like serine proteases have been identified from *F. oxysporum* with highest activity at pH 8.0 and was active in pH range of 7.0 to 9.0 (Rodríguez *et al*., 2017).

In the present work, specific activity of *Fusarium* isolates fall in very wide range in *in vitro* condition. We have found that specific activity of *Fusarium*

isolates was ranged from 223.68±18.83 units/mg to 24.02±0.56 units/mg of protein. One study has reported the specific activity of 124 units/mg from culture filtrate and 1759 units/mg from concentrated culture filtrate of *F. solani* with optimum pH of 8.0 (Bhuvaneswari and Balasubramanian, 1999). Rodarte and group has reported the specific activity of 37.4 units/mg to 37.5 units/mg of protease from *F. solani* and *F. moliniformae*, respectively with optimum pH of 9.0 which is in the range of specific activity we have obtained (Rodarte *et al*., 2011). The specific activity of proteases of FSSC isolates was high in pH range of 3.0-7.4 but the optimum pH was 7.4 except for few isolates which were having optimum pH of 3.0, these results indicates majority of proteases produced by these *Fusarium* isolates are neutral to acidic. There are reports which shows optimum pH of 8.0 & 9.0 for *F. solani* protease (Al-Askar *et al*., 2014; Bhuvaneswari and Balasubramanian, 1999; Rodarte *et al*., 2011).

Extracellular proteases produced by FSSC isolates were inhibited by all 3 inhibitors, EDTA, PMSF and Pepstatin A while proteases produced by *F. delphenoides* isolates were inhibited by EDTA and are therefore metalloproteases. Proteases from *F. solani* were inhibited with PMSF and EDTA in gelatine zymography (Gopinathan *et al*., 2001). Presence of metalloprotease in *F. solani* by gelatine zymography has also been reported (Dong *et al*., 2004). Presence of metalloproteases genes and serine protease genes in *F. solani* were reported (Iqbal *et al*., 2018). Inhibition of *F. solani* protease by PMSF had been shown (Bhuvaneswari and Balasubramanian, 1999). Our results comply with literature. However, none of the studies document the inhibition of FSSC protease with Pepstatin A, and in present study, we were able to see inhibition of FSSC protease with Pepstatin A in azocasein assay as well as gelatine zymography. In some of the isolates, increased activity was observed in presence of PMSF, EDTA and Pepstatin A. Several reports have shown that EDTA, Pepstatin A, DTT, Iodoacetamide and beta mercaptoethanol increase activity of protease enzyme but the reason for increase in the enzyme activity in presence of inhibitor is not well understood and needs to be investigated (Bhuvaneswari and Balasubramanian, 1999; El-

Khonezy *et al*., 2015; Li *et al*., 2007; Sharma and De, 2011; Wang *et al*., 2006).

All FSSC isolates in the present work, produced more than one kind of protease and it was apparent that serine and metalloproteases are among the important ones in FSSC. Based on this initial information, protease genes ranging from 50-100 kDa were selected from the whole genome sequence of *N. haematococca* to study the expression of these genes in both, an *in vitro* and an *ex vivo* explant infection condition.

In the present study, an *ex vivo* corneal model of *Fusarium* keratitis using caprine cornea was developed and expression of certain virulence genes was evaluated. First corneal cultures using human cornea organ cultures were reported in 1977 to study the epithelial-endothelial interactions. The sterilization was done using penicillin, streptomycin and mycostatin containing MEM medium. The dissected corneas were placed in free floating petri plate and incubated at 37ºC in 5% CO_2 containing air. At the interval of 3 days, media was changed. H-E stain was used to monitor the interaction (Yanoff and Cameron, 1977). Later, Richard and group developed the air/liquid corneal organ culture to study wound healing. The authors called the model as air/liquid because it mimicked an *in vivo* situation where the epithelium was exposed to air while the endothelium faces the liquid (aqueous humor). In above studies, cornea was maintained in media for three weeks without edema and other structural changes (Richard *et al*., 1991)**.** Similar air/liquid rabbit corneal organ culture was used to study the effect of topical human amniotic fluid (HAF) and equine amniotic fluid (EAF) on corneal re-epithelialization and stromal wound healing. The eye globe of rabbit was maintained in DMEM medium with antibiotic and antimycotic solution and media was change after 36 hrs (Castro-Combs *et al*., 2008). Rabbit *ex vivo* corneal organ culture has been used to study herpes simplex keratitis. To maintain the corneal shape 1% agarose solution was added to corneas placed in spot plate facing endothelial layer towards air. After solidification of artificial bead, the corneas were reversed. The authors reported that corneas might be cultured for one week with media change over 48 hrs using this method. (Alekseev *et al*., 2012). Similar model has also been used to evaluate

the anti-scarring therapies (Sriram *et al*., 2014). In above mentioned studies, the histology of cornea was done using H-E staining after embedding in paraffin. Human *ex vivo* corneal model has been developed to study the hyphal penetration *F. oxysporum* during keratitis. The corneas were maintained in supplemented hormonal epithelial medium (SHEM), consisting equal amount of DMEM medium and Ham's F12 medium supplemented with insulin, epidermal growth factor, transferrin, hydrocotison, cholera toxin A, sodium selenite, FBS, gentamycin and DMSO. Corneas were scarified artificially and inoculated with $1X10^5$ CU of *F. oxysporum*. The media was changed after 24 hrs. The sections of corneas were stained with periodic acid-Schiff (PAS) reagent for histology. (Hua *et al*., 2010). To study the molecular mechanism of *C. albicans* adherence and drug screening on corneal surface, a mice *ex vivo* corneal organ culture was developed. The corneas were inoculated with 10^7, 10^8 and 10^9 spores of *C. albicans*. For corneal histology, H-E stain and calcofluor white stain were used (Zhou *et al*., 2011). In all these models, the corneas were maintained in static condition. Deshpande and group has reported use of rocking media instead of static media for study of effect of proinflammatory cytokines on wound healing, in which corneas survived for up to 4 weeks (Deshpande *et al*., 2015). In the present study also goat cornea was viable for two weeks.

Our model is similar to the air/liquid model with few changes. First, the cornea used was goat cornea and second, the cornea was placed on a solid agarose-gelatine support which helped to cornea to remain well shaped. The artificial corneal buttons were made and placed in sterile 12 well tissue culture plate, upon it the dissected sterile corneas were placed facing epithelial surface upward. Later the solid support was prepared using method of (Alekseev *et al*., 2012). The advantages of using the goat cornea are that no ethical clearances required, the cornea is large and comparable to humans and they can be procured easily from slaughter house. *Ex vivo* models have been used to study keratitis caused by *Candida*, *Fusarium* and Herpes simplex virus. In the present study also goat cornea was viable for two weeks which was measured using trypan blue assay and MTT assay. On the basis of an *in vitro* results, three isolates were selected (Cs1, Cc50 and CSH3) for an *ex vivo*

infection model and expression of protease genes was quantified using qRT-PCR. The current study emphasizes the importance of using convenient *ex vivo* model systems to study expression of many virulence genes altogether.

We found three interesting findings in the present study (i) Different protease genes show expression during an *in vitro* growth and an *ex vivo* corneal explant conditions. For e.g. proteases (C7YY94, C7Z7U2, C7Z6W1) expressed during an *in vitro* growth while, proteases (C7Z6W1, C7YVF3) expressed during an *ex vivo* corneal explant infection (ii) Carboxypeptidase (C7YVF3) was identified in HR-LCMS/MS and it was also highly expressed in the corneal infection. (iii) Aminopeptidase (C7Z6W1) showed around 50-fold up-regulation during corneal infection. We couldn't verify the presence of aminopeptidase (C7Z6W1) in mass spectrometry analysis but, another tripeptidyl aminopeptidase (TPP) that showed homology to a TPP from *F. langsethiae* (A0A0M9ESD1) was detected. We also found another two serine peptidases which showed homology to Q59RK9 (*C. albicans*) and A6ZRW1 (*S. cerevisiae*) using mass spectrometry. When these proteins where blasted against the *N. haematococca* genome; proteins matching with 49.5% and 32.47% similarity were found, which are predicted and yet to be annotated.

The first report on the role of proteases in collagen destruction was shown in rabbit keratitis (Dudley and Chick, 1964). Later, characterization of proteases from *A. flavus* and *F. solani* was carried out using an *in vivo* rabbit model of keratitis. Researchers found out that 200kDa, 92kDa and 58kDa gelatinases were found in infected cornea with both *A. flavus* and *F. solani*. Also, 65kDa protease was found in all infected and non-infected eyes. These gelatinases showed inhibition with EDTA and was considered as Metalloproteases (Gopinathan *et al*., 2001; Matsubara *et al*., 1991). Our results also corroborate with their findings as proteases of molecular weights of around 100kDa and 50-60 kDa were found in zymography. We believe that the high molecular weight band found in zymography may correspond to the proteases C7Z7U2/C7ZNV5 and the low molecular weight bands may correspond to proteases-C7YVF3 and C7Z6W1. Our study warrants the need to characterize these unannotated genes using gene deletions and over expression studies to

validate the role of these proteases as a promising virulence factor. It is also apparent that information regarding virulence of FSSC is meager in spite of FSSC being the most prevalent pathogen in the genus.

Differences in the expression of genes in an *in vitro* and an *ex vivo* corneal explant infection suggest that such differential expression is required for the survival in the corneal tissue. A similar report was shown for the CtsD protease, which showed expression in an *in vivo Galleria mellonella* infection but not during an *in vitro* growth (Lee and Kolattukudy, 1995; Vickers *et al*., 2007). It can be concluded that certain proteases (C7Z6W1 and C7YVF3) are essential for infection.

Secretome and exoproteome analysis using mass spectrometry is a novel approach to identify factors responsible for virulence of pathogenic fungi. Secretome analysis of *F. graminerum* has been carried out to identify proteins which could be possible virulence factors and might play a role during *F. graminearum* infection (Ji *et al*., 2013). *A. flavus* isolated from infected cornea, sputum and a saprophyte were pooled and identified using high resolution mass spectrometry in order to get the total exoproteome from cultures isolated from different sources (Selvam *et al*., 2015). The exoproteome analysis of a keratitis causing *A. flavus* showed that nearly 50% of the exoproteins possess catalytic activity and one of these, an alkaline serine protease (Alp1) is abundant and present in multiple proteoforms (Leema *et al*., 2010; Selvam *et al*., 2015). Novel hydrolyting enzymes have been identified by Quantitative iTRAQ Secretome analysis of *A. niger* (Adav *et al*., 2010). However, studies of exoproteome or secretome analysis of pathogenic *Fusarium* species causing keratitis are lacking. Here we attempted exoproteome analysis from keratitis causing pathogenic *F. solani*. Protein identification data here shows that in exoproteome analysis peptide sequences matches with *S. cereviseae*, *C. albicans*, *Pseudomonas* and *N. haematococca*. When peptide search was done with only *N. haemotococca*, we were not able to get enough number of proteases but when peptide sequences were blasted with other fungal and bacterial genome we were able to find proteases. It appears that these proteases sequences are still un-annotated in *N.*

haematococca reference genome database and need to be identified and reported in *N. haematococca* database. We were able to find that some of the proteases in exoproteome which do not possess signal peptide sequence but are being secreted in extracellular medium by fungi. Similar results have been reported by selvam and group (Selvam *et al*., 2015), where exoproteome analysis of *A. flavus* has shown that only 50% of proteins possess signal peptide sequence and remaining proteins are being secreted by non-classical pathway. The protein database and whole genome database of other *Fusarium* spp. like *F. graminerum*, *F. oxysporum* is well characterized and there are several reports of protein identification in exoproteome analysis of these species. Phalip and group has reported exoproteome analysis of *F. graminerum* where fungi was grown on plant cell wall and the type of enzymes secreted were identified. They have reported that 9% of proteins from whole exoproteome were having peptide hydrolysing activity (Phalip *et al*., 2005). Ji and group has reported that 17% of proteins were having peptide hydrolase activity in exoproteome of *F. graminerum* (Ji *et al*., 2013). The unavailability of a well curated database for mass spectrometry analysis of *N. haematococca* proteins is impacting on our understating and characterization of the proteases from *Fusarium* isolates.

Mass spectrometry analysis revealed the presence of two important proteases, a tripeptidyl peptidase and a carboxypeptidase. Tripeptidyl peptidase belongs to sedolisin family which is sub-family of subtilisin serine protease. In *A. fumigatus*, SedB, SedC, SedD and SedA are known to degrade the proteins and provide nutrition to fungus during infection. Tripeptidyl peptidases are also involved in degradation of bone matrix protein which is made up of collagen (Reichard *et al*., 2006; Vines and Warburton, 1998). Tripeptidyl peptidase presence has been reported in *S. lividans*. Carboxypeptidases have been reported as virulence factors in number of pathogens. It has been reported that carboxypeptidase along with subtilisin like Pr1 is required by *Metarhizium anisopliae* for peptide degradation during pathogenesis. Carboxypeptidase REP34 is required by *Francisella tularensis* for invasion in to host tissue and contribute to virulence (Feld *et al*., 2014; Stleger *et al*., 1994). In *Porphyromonas gingivalis* and *Trichophyton rubrum*

carboxypeptidase along with aminopeptidase is required for to obtain amino acid for its growth during pathogenesis (Masuda *et al*., 2002; Zaugg *et al*., 2008).

To summarize, FSSC isolates causing keratitis produce varying amounts and types of proteases during growth and infection. Based on qRT-PCR and mass spectroscopic results it can be concluded that both, carboxypeptidase and aminopeptidase contribute to pathogenesis of FSSC.