Chapter 4

Quantification and characterization of cell wall component beta glucan and expression analysis of beta glucan synthase and beta glucanase

4.1 Introduction:

Fungal cell wall consists of three main components; lipids, proteins and carbohydrates (glucan, mannan and chitin). Cell wall provides protection from host and environmental stress and is also important to maintain the shape (Samalova *et al*., 2017). For the proper architecture of fungal cell wall, polysaccharides mainly beta glucan, alpha glucan, mannan and chitin, and proteins mainly galactomannose protein, GPI anchored and surface proteins are required (Free, 2013; Yoshimi *et al*., 2017).

The carbohydrate content of the cell wall varies depending upon the type of fungal species. β-glucans typically comprise 30-80% of the cell wall polysaccharide content (Free, 2013). Increased virulence with increased chitin and phospholipid along with glucan-containing covalently attached phospholipid are characteristics of highly virulent strains (Brass *et al*., 1982). Different types of glucan found in fungal cell wall are α-glucan, 1,3-β-glucan, 1,6-β-glucan, 1,3-1,6-β-glucan, 1,3-1,4-β-glucan and 1,4-α-glucan (Bernard and Latgé, 2001; Bowman and Free, 2006; Grün *et al*., 2004; Klis *et al*., 2001).

α-glucan: α-glucan seems to mask components of the cell wall in *B. dermatitidis* such as WI-1 antigenic adhesin on the surface of the yeasts and this adhesin is associated with induction of humoral immune response and macrophage activation. 1,3-α-glucan of *P. brasiliensis* protects the fungus against digestive enzymes of the host leukocytes and macrophages.

β-glucan: They have a common structure comprising a main chain of (1,3)-linked β-D glucopyranosyl units along with randomly dispersed side chains of β-D-glucopyranosyl units attached by (1,6) linkages.

In the present work, total β-glucan and 1,3-β-glucan was quantified from *Fusarium* isolates. Quantitative expression of genes contributing to β-glucan synthesis was done for FSSC isolates and immunomodulatory effects of mycelia and extracted β-glucan was studied.

4.2 Materials and Methods:

4.2.1 Growth of fungi:

All isolates were first grown on PDA. 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 and grown in PDB for 10 days at 30ºC without shaking. At the end of 10^{th} day the mycelial mass was separated from culture filtrate using autoclaved whatman no.1 filter paper.

4.2.2 Extraction of beta glucan:

β-glucan was extracted using method described by Ohno and group (Ohno *et al*., 1999). 10 day old fungal mycelial mat after filtration, was washed with D/W and water was decanted. Mycelial mat was oven dried. The dried fungal mat was crushed with the help of pestle. One gram of dried mycelial mass was suspended in 12.5 ml of 0.1M NaOH (Merck, USA) and 100 ml of sodium hypochlorite (Merck, USA). Incubation was carried out at 4ºC for 24 hours followed by filtration through muslin silk to separate insoluble fraction. The insoluble fraction was washed with D/W and suspended in chilled ethanol and kept at -20ºC for 10 minutes. After incubation, ethanol was decanted by filtration. The insoluble fraction was re-suspended in chilled acetone (Merck, USA) and kept at -20ºC for 10 minutes. The insoluble fraction was recovered by decanting the acetone through filtration. The recovered insoluble fraction was macerated with 10 ml of dimethyl sulphoxide (DMSO) (Merck, USA) to extract β-glucan. Ultra-sonication was carried out at 500W, 20 kHz. Insoluble fraction in DMSO was incubated in water bath for 60 minutes at 90˚C. After first 20 minutes, sonication was carried out on ice for 3 minutes and again incubated for 20 minutes. This step was repeated total 3 times. After sonication, centrifugation was carried out at 1,000xg at RT for 15 minutes to remove the insoluble fraction. The supernatant was transferred to another tube and four volumes of ethyl alcohol was added and centrifuged at 5,000xg for 10 minutes at RT. The pellet contained crude β-glucan, was dissolved in 1ml DMSO for further use.

4.2.3 β-glucan quantification:

Quantification of β-1,3-glucan was carried out with Enzyme linked immunosorbant assay (ELISA) and quantification of total β-glucan was carried out with Congo red assay.

4.2.3.1 ELISA:

ELISA was carried out for quantification of β-1,3-glucan . Primary antibodies were specific to β-1,3-glucan . Standard β-glucan and extracted β-glucan were diluted in 1X PBS and then used for assay. Standard concentration range used was 0.1-100 µg/ml. 96 well polyvinyl chloride (PVC) plates (Thermo scientific, USA) were used to carry out assay. 1X PBS was used as blank. 100 µl of all standard dilutions and samples were loaded in plate and plate was kept in moist chamber at 4ºC overnight. After incubation, plate was decanted by inverting and plate was tapped 2-3 times on blotting paper to remove any solution. Washing was done for 3 times with 200 µl of 1X PBS buffer for 3 minutes for each wash. The remaining protein binding sites of coated wells were blocked by addition of 200 µl of blocking buffer (5%) containing 1 gm skim milk (Himedia, India) in 20 ml of 1X PBS. The plate was incubated at RT for 2 hours. The blocking buffer was removed by flicking the plate over sink. The plate was washed 2 times with 200 µl of 1X PBS buffer for 3 minutes for each wash. Primary antibody (BioSupplies, Australia) was diluted 1:1000 in 5% blocking buffer freshly and 100 µl was added in to each well and plate was incubated at RT for 2 hours. Primary antibody was removed by flicking the plate over sink. The plate was washed 4 times with 200 µl of 1X PBS buffer for 3 minutes for each wash. Horse reddish peroxidase conjugated secondary antibody was diluted 1:2000 in 5% blocking buffer freshly and 100 µl was added in to each well and plate was incubated at RT for 2 hours. Secondary antibody was removed by flicking the plate aver sink. The plate was washed 4 times with 200 µl of 1X PBS buffer for 3 minutes for each wash. 100 µl of TMB (Sigma-Aldrich, USA) solution was added to each of coated wells, incubated for 15-30 minutes for development of blue colour. 100 µl of 2M H_2SO_4 (Merck, USA) was added to each well and yellow colour was developed. The optical density was measured at 450 nm in ELISA plate reader

(Multiscan Go, Thermo scientific, USA). The standard curve was plotted with concentration on X axis and absorbance on Y axis.

4.2.3.2 Congo red assay:

Congo red (Merck, USA) solution was made in deionised water at concentration of 1 mg/ml. Standard β-glucan (1mg/ml) concentration range used was 20-200 µg/ml. 20 µl of extracted crude β-glucan in DMSO was taken in tubes. 10 µl of Congo red was added to each tube and final volume was made up 1000 µl using deionised water. All the tubes were incubated at RT for 3 hours. The optical density was measured at 523 nm using spectrophotometer (Multiscan GO, Thermo scientific, USA).

4.2.4 Fluorescence Microscopy:

Aniline blue (Himedia, India) fluorescence dye was used which binds specifically to β-1,3-glucan. Fungal isolates were grown on PDA for 7-8 days. Small amount of mycelia was taken on microscopic slide and stained with 0.05% aniline blue made in 0.67M KH_2PO_4, pH 8.5.After 10 minutes excess dye was washed with 0.67M KH_2PO_4, pH 8.5. (Evans *et al*., 1983). For fluorescence analysis, specimens were examined with a Nikon eclipse 80 I microscope equipped with epifluorescence optics and Nikon plan fluar objectives. Illumination was provided by Nikon super high pressure mercury lamp. Maximum fluorescence intensity is obtained using exciter filter BG12 and barrier filter 53 after aniline blue staining.

4.2.5 Characterization of β-glucan:

Characterization was primarily carried out by thin layer chromatography (TLC) and further it was characterized using Fourier Transform Infrared (FT-IR) spectroscopy.

4.2.5.1 Thin Layer Chromatography:

Crude β-glucan extracted from fungal isolates was primarily characterized with respect to standard β-glucan from fungus *Euglena gracilis* (Sigma-aldrich, USA). TLC was carried out as described by Komaniecka and Choma, 2003. TLC plates (Kieselgel 60, E. Merck, USA) were cut in to piece of 10x2 cm. It was marked with pencil 1 cm from bottom end and 1 cm from upper

end. Spots were marked with pencil at distance of 1 cm. TLC chamber was saturated with solvent system for 30 minutes before running TLC. Two different solvent systems were used to characterize β-glucan. Solvent system A contained n-butanol (Merck, USA): ethanol: D/W (5:5:4) (V/V) and solvent system B contained n-propanol (Merck, USA): D/W (8:3) (V/V). 10µl of 1mg/ml standard β-glucan and 10µl of extracted β-glucan were applied to marked spots plate was placed carefully into the TLC chambers in vertical position. As soon as the solvent reached to marked line at distance of 8 cm, TLC plate was removed from TLC chamber and air dried. For development of TLC plate, iodine crystals (Merck, USA) were filled in clean glass beaker and covered with aluminium foil and allowed to form vapours. TLC plate was placed in Iodine contained beaker until brown spots were visible and then plate was removed from beaker. R_f value was calculated from TLC plate using standard formula.

4.2.5.2 FT-IR (Fourier Transform Infrared) spectroscopy:

FTIR was carried out as described by Xia and group (Xia *et al*., 2014). FT-IR spectroscopy was carried out to explore the structural features of extracted β-glucan along with standard 1,3-β-glucan from fungus *Euglena gracilis* (Sigma-Aldrich, USA), Laminarin (1,3-1,6-β-glucan) from *laminaria digitata* (Sigma-Aldrich, USA) and Curdlan (Sigma-Aldrich, USA). 10 mg of β-glucan extracted from fungal isolates as well as standard β-glucan was homogenised with 1gm KBr (Potassium bromide) (Merck. USA) in mortar pestle for 5 minutes and placed in sample holder and pressed to form a pellet. This was placed in to FTIR spectrophotometer (PerkinElmer Spectrum II, India) and scanned at wavelengths of 4,000-400 cm^{-1} with resolution range of 4 cm^{-1}. The spectrum was recorded with software PerkinElmer Spectrum version 10.03.06 and spectra was generated with help of e-FTIR software.

4.2.6 RNA isolation: (mentioned in chapter 3)

4.2.7 cDNA synthesis: (mentioned in chapter 3)

4.2.8 Primer designing for Glucan synthase and Glucanse genes:

Primer designing for Glucan synthase and glucanse genes was done for *N. haematococca*, as whole genome data is available for *N. haemoatococca*. Genes for glucan synthase and glucanse were selected from NCBI. Total 8 genes were found in *N. haemoatococca*. Six genes belonged to glucanse and 2 genes belonged to glucan synthase. As a housekeeping gene, 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 4.1).

4.2.9 Quantitative RT-PCR (qRT-PCR):

Quantitative RT-PCR (qRT-PCR) was carried out as mentioned earlier. The PCR cycle included following steps: 1 cycle of 95ºC for 3 minutes, 40 cycles of 95ºC for 30 seconds, 60ºC 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.

4.2.10 Immunomodulatory effect of β-glucan from *Fusarium* spp. on human whole blood.

Whole blood assay was carried out as described by Noss and group (Noss *et al*., 2013). Curdlan (β-1,3-glucan), Laminarin (β-1,3-1,6 glucan) (1mg/ml) and extracted fungal β-glucan (1mg/ml) were re-suspended in RPMI 1640 medium (Himedia, India) supplemented with 100 I.U./ml penicillin and 100 mg/ml streptomycin (Gibco, Thermo scientific, USA). 10^6 Heat killed *E. coli* cells were used as positive control. 100 µl of standard curdlan, 100 µl of laminarin, 100 µl of extracted fungal β-glucan , 100 µl of NaOH treated fungal mycelia re-suspended in PBS , 100 µl of fungal mycelia suspended in PBS, 100 µl PBS and 100 µl of 1M NaOH were added to sterile flat bottom 96-well cell culture plates. 100 µl of heparinized blood was added to each well and incubated in CO_2 incubator at 5% CO_2 and 37ºC for 24 hrs. After incubation, plate was centrifuged at 1000Xg for 5 minutes and supernatant was taken in to sterile tubes and used for TNF alpha estimation using BDopt EIA Human TNF alpha ELISA kit (BD scientific, USA).

Table 4.1: The genes investigated in the current study, their known functions, and the primers used for quantitative real-time polymerase chain reaction.

No	Gene ID	Name	Primer sequences (5'—›3')
		Glucanses	
1 (G1)	NECHADRAFT_95637	Hypothetical protein	F: TGACGGCAAGCTGGTGTTTA R: CTGCGTAGTAAGCCTCCACC
2 (G2)	NECHADRAFT_123099	Hypothetical protein	F: GGTGGCGAGTCTCAACAAGA R: GAGGGCCGAACAACAACAAC
3 (G3)	NECHADRAFT_94692	Hypothetical protein	F: AGGCGAAGAACTCGTCTGTG R: GCGGCTGTAGTGGTTTGTTG
4 (G4)	NECHADRAFT_106067	Glycoside hydrolase	F: CTCAGGCTACTTCGCTCCAG R: GATGTTCTCACCGGCAGTCA
5 (G5)	NECHADRAFT_68445	Hypothetical protein	F: CTGGAACAACAACCCCGAGA R: CTACTCAAGCGAGGAAGGGC
6 (G6)	NECHADRAFT_ 63674	Hypothetical protein	F:CATGGCAATGGAATGGCGAG R:CAGATTCATCCCAGACGGCA
		Glucan synthases	
7	FKS 1	glycosyltransferase family	F: GCCCAAGCTCTACTCCGTTT R: GGATGTACTCGCCACGGTAG

8 (GS2)	NECHADRAFT_88969	Hypothetical protein	F: CGGACCCGTTGTTTCCTACA R: GGCGTAGACCTGACACACAA
		House-keeping gene	
9	TEF1	Translation elongation factor 1 alpha	F: TTCAAGTGGGCGATGCTCTT R: AGTTGATGGGGTCTGCTGTG

4.2.11 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.

4.3 Results:

4.3.1 Yield of crude β-glucan from dried mycelium:

The initial dry weight of the fungal biomass was measured prior to start the extraction. After extraction the weight of crude extracted β glucan was measured in mg/gm of the dry weight of fungus. The yield of crude β glucan was then converted in terms of % (w/w) which ranges from 4.0-81.5% w/w in all *Fusarium* isolates. Table 4.2 shows the initial dry weight of all *Fusarium* isolates along with yield of crude β-glucan. In FSSC isolates, the crude β-glucan yield was in the range of 4.0-20.8% w/w in CSH11 and Cc240, respectively. In FDSC and *F.sacchari* isolates β-glucan yield was highly variable. In FDSC isolates, crude β-glucan yield was 80% w/w, 13% w/w and 7.8% w/w, and in isolate Cc26, Cc119 and CSH4, respectively. In *F. sacchari* isolates, crude β-glucan yield was 4.2% w/w, 7.1% w/w, 31.6% w/w and 81.5 % w/w in Cc52, Cc61, Cc167 and Cc215, respectively. In *F. equiseti* isolate Cc172 the yield of crude β-glucan was 7.8% w/w. The crude extracted β-glucan was dissolved in DMSO for further quantification.

Table 4.2: Initial fungal dry weight and yield of crude β-glucan extracted from *Fusarium* isolates.

Fungal isolates	Initial Dry weight (gm)	Dry weight of β-glucan (mg)	Conc. (mg/gm)	Yield of β-glucan (%w/w)
FSSC isolates				
CSH1	0.52	40	76.92	7.6
CSH2	0.71	60	84.50	8.4
CSH3	1.63	204	125.15	12.5
CSH5	1.05	90	85.71	8.5
CSH6	0.79	50	63.29	6.3
CSH7	0.88	80	90.90	9.0
CSH8	0.77	79	102.59	10.2
CSH9	1.1	70	63.63	6.3
CSH10	0.85	47	55.29	5.5
CSH11	0.84	34	40.47	4.0
Cs1	0.59	50	84.74	8.4
Cs2	0.61	70	114.75	11.4
Cc50	0.53	40	75.47	7.5
Cc240	0.67	140	208.95	20.8
FDSC isolates				
Cc26	0.05	40	800	80
CC119	0.138	18	130	13
CSH 4	0.38	30	78.94	7.8
***F. sacchari* isolates**				
Cc52	0.19	80	421.05	4.2
Cc61	0.7	50	71.42	7.1
Cc167	1.61	510	316.77	31.6
Cc215	0.601	490	815.30	81.5
***F. incarnatum-equiseti* isolate**				
Cc172	0.671	53	78.98	7.8

4.3.2 Quantification of β-glucan:

In the present study, Enzyme linked immune sorbent assay (ELISA) and Congo red assay were performed to estimate the concentration of β-1,3-glucan and total β-glucan from clinical *Fusarium* isolates.

4.3.2.1 Enzyme linked immunosorbant assay (ELISA):

Concentration of β-(1,3)-glucan in *Fusarium* spp. was ranging from 0.03±0.00 mg/gm to 63.04±7.92 mg/gm in *F. sacchari* Cc61 and FDSC Cc26, respectively. Table 4.3 shows the concentration β-1,3-glucan in *Fusarium* isolates. In FSSC isolates, the maximum β-1,3-glucan concentration was found to be 37.07±1.72 mg/gm in Cc50 and minimum β-1,3-glucan concentration was found to be 0.10±0.08 mg/gm in Cc240. The β-1,3-glucan concentration other FSSC isolates was in range of 0.82±0.09 mg/gm to 3.71±1.64 mg/gm. In FDSC isolates, lowest β-1,3-glucan was found in Cc119 (0.89±0.39 mg/gm) and highest in Cc26 (63.04±7.92 mg/gm). In *F. sacchari* isolates, β-1,3-glucan was found lowest in isolate Cc61 and highest in isolate Cc167 which was 0.03±0.00 mg/gm and 11.50±0.41 mg/gm, respectively.

4.3.2.2 Congo red assay:

Total β-glucan was quantified using Congo red assay. Congo red estimation showed variation in the amount of total β-glucan among different *Fusarium* isolates and even in the isolates of same species. Among all *Fusarium* isolates, total β-glucan was found to be maximum in FDSC Cc26 (361.31±8.32 mg/gm) and minimum in FSSC Cs1 (8.50±0.35 mg/gm). Table 4.3 shows the concentration of total β-glucan in *Fusarium* isolates. In FSSC isolates, the estimated concentration of total β-glucan was lowest in Cs1 (8.50±0.35 mg/gm) and highest in Cc240 (54.42±1.55 mg/gm). In FDSC isolates, lowest total β-glucan was found to be in isolate CSH4 (21.20±0.75 mg/gm) and highest in Cc26 (361.31±8.32 mg/gm). In *F. sacchari* isolates, total β-glucan was found to be lowest in Cc 61 and highest in Cc 215 which was 11.30±0.96 mg/gm and 152.56±2.11 mg/gm, respectively.

Table 4.3: Quantification of β-1,3-glucan and total β-glucan in *Fusarium* isolates using ELISA and Congo red assay.

Fusarium Isolates	β-1,3-glucan (mg/gm)	Total β-glucan (mg/gm)
FSSC isolates		
CSH 1	1.50±0.14	17.91±1.12
CSH 2	1.19±0.52	16.86±0.63
CSH 3	0.87±0.13	30.17±0.39
CSH 5	0.73±0.21	20.84±1.26
CSH 6	0.98±0.60	20.77±0.69
CSH 7	0.96±0.3	23.85±0.77
CSH 8	1.51±0.27	25.40±1.46
CSH 9	1.31±0.14	25.75±1.46
CSH 10	0.82±0.09	16.62±0.17
CSH 11	3.71±1.64	34.06±1.26
CS 1	1.88±0.86	8.50±0.35
CS 2	2.67±1.07	18.44±0.76
Cc50	37.07±1.72	42.43±1.30
Cc240	0.10±0.08	54.42±1.55
FDSC isolates		
Cc26	63.04±7.92	361.31±8.32
Cc119	0.89±0.39	136.75±2.51
CSH 4	2.00±0.47	21.20±0.75
***F. sacchari* isolates**		
Cc52	10.45±3.55	136.75±2.63
Cc61	0.03±0.00	11.30±0.96
Cc167	11.50±0.41	25.56±1.03
Cc215	0.64±0.09	152.56±2.11
***F. incarnatum-equiseti* isolate**		
Cc172	0.36±0.14	63.28±1.13

4.3.3 Fluorescence Microscopy:

Aniline blue staining was carried out for those isolates which showed highest and lowest β-1,3-glucan concentration which were Cc26 (FDSC) and Cs1 (FSSC). Both of the isolates showed presence of fluorescent aniline blue-β-glucan complexes. The fluorescence intensity was higher in Cc26 and was lower in Cs1 (Figure 4.1). This result is in accordance with quantification data of β-1,3-glucan estimation by ELISA method.

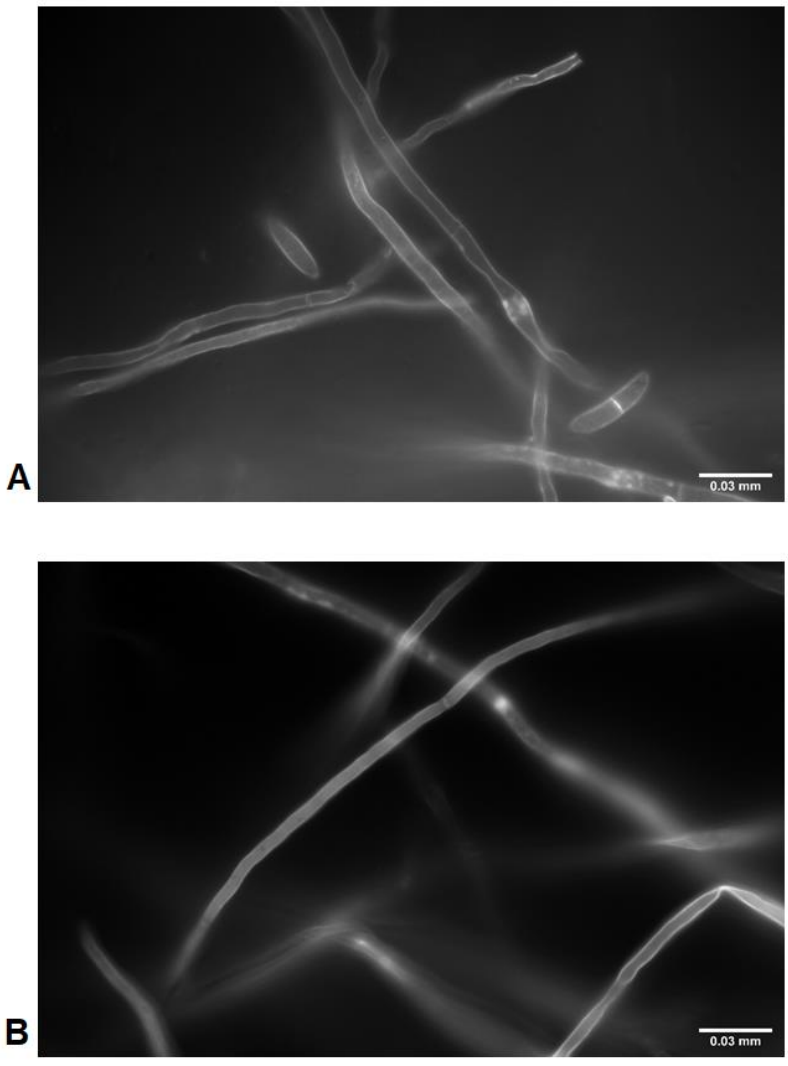

Figure 4.1: Light micrograph of *Fusarium* mycelia stained with aniline blue. (A) FSSC Cs1 and **(B)** FDSC Cc26. Both the hyphae readily bound to the aniline blue stain demonstrating the presence of β-1,3-glucan residues on their surfaces. Fluorescence intensity is higher in FDSC Cc26 than FSSC Cs1.

4.3.4 Characterization of β-glucan:

β-glucan characterization was done by using TLC primarily followed by FTIR analysis. For characterization purpose, β-1,3-glucan from *E. gracilaris*, Curdlan and Laminarin from *L. digitata* were used as standard β-glucan.

4.3.4.1 Thin layer chromatography:

Initially to characterize the extracted β-glucan from *Fusarium* isolates, TLC was carried out along with standard β-glucan. TLC was carried out with one representative isolate from each group, FSSC CSH1, FDSC Cc26 and *F. sacchari* Cc52 and standard β-1,3-glucan from *E. gracilaris*. After development of plate with iodine vapour, a single brown spot of β-1,3-glucan was observed with R_f value of 0.83. The extracted β-glucan also gave similar spots with similar R_f value but the spots were larger which might be possible because of more amount and various type of β-glucan might present in sample. Figure 4.2 shows the TLC plate of Standard β-1,3-glucan and glucan extracted from CSH1, Cc26 and Cc52. After primary indication and characterization of β-glucan, further characterization was done using FTIR analysis for all *Fusarium* isolates.

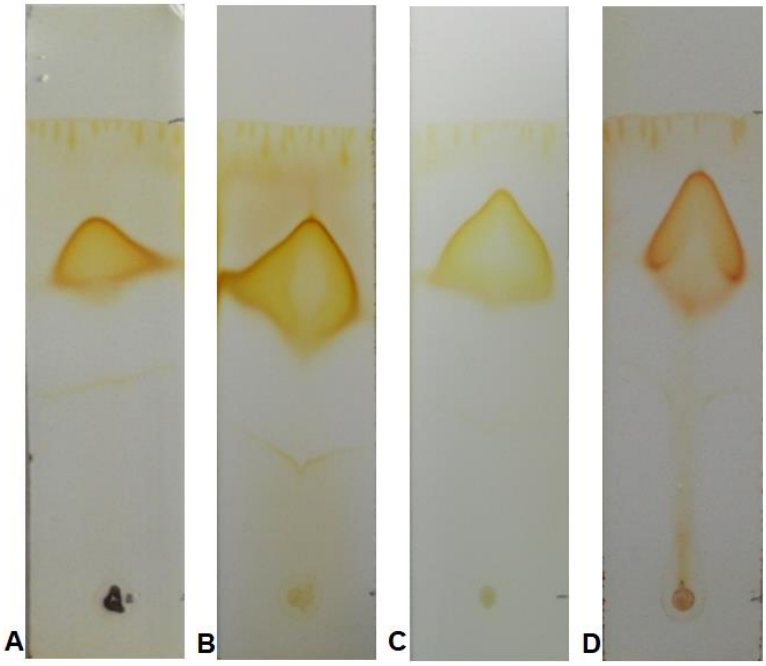

Figure 4.2: Representative TLC plate of Standard β-1,3-glucan and crude β-glucan extracted from *Fusarium* isolates. (A) β-1,3-glucan from *E. gracilaris* **(B)** FSSC CSH1, **(C)** FDSC Cc26 and **(D)** *F. sacchari* Cc52. Brown spot represents β-glucan and rf value is 0.83.

4.3.4.2 FTIR analysis:

Fourier-transform infrared (FT-IR) spectroscopy was carried out to explore the structural features of extracted β-glucan along with standard β-glucan. Figure 4.3, Figure 4.4 and Figure 4.5 shows the FTIR spectra of standard β-1,3-glucan, curdlan and Laminarin (β-1,3-1,6-glucan). Spectra was recorded from 400-4000 cm^{-1}.

Figure 4.6 to Figure 4.24 shows FTIR spectra of FSSC isolates, CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50 and Cc240, respectively. Figure 4.25 to Figure 4.28 shows FTIR spectra of FDSC isolates, Cc26, Cc119 and CSH4. Figure 4.29 to Figure 4.33 shows FTIR spectra of *F. sacchari* isolates, Cc52, Cc61, Cc167 and Cc215. Figure 4.34 and figure 4.35 shows FTIR spectra of *F. incarnatum-equiseti* Cc172.

The region between 3000-4000 cm^{-1} contain a broad peak which corresponds to hydroxyl group (-OH). Depending upon type of polysaccharide, the peak position differ. Particularly, 3400-3451 cm^{-1} peak position corresponds to -OH stretch. The peak in this region was present in majority of isolates. However, in some cases the peak was shifted a little and give absorbance below 3400 cm^{-1} which can be seen in spectra of Laminarin, CSH4, Cs1 and Cc240, which gave peak at 3396.41 cm^{-1}, 3224.0 cm^{-1}, 3397.7 cm^{-1} and 3378.41 cm^{-1}, respectively. The weak stretching in region of 2915-2940 cm^{-1} is characteristic of -CH bond. The peak in this region was observed in all *Fusarium* isolates which indicates presence of -CH bond in all isolates. The presence of polysaccharide in present study is depicted by occurrence of absorption band in region of 950-1200 cm^{-1}, which can be seen spectra of all isolates. The peak in the region of 1144-1162 cm^{-1} corresponds to C-O bond. The peak in the region of 1612-1644 cm^{-1}, 1748 cm^{-1} and 1378 cm^{-1} corresponds to -C=O bond. Presence of peak at and near to the position 1318 cm^{-1}, 1420 cm^{-1}, 1450 cm^{-1}, 1616 cm^{-1} and 1627 cm^{-1} indicates presence of -COO group. Absorption peak at 1156-1165 cm^{-1} indicate linear structure of β-D-glucan linked through 1—›3 linkage. The absorption peak in and near the region of 875-901 cm^{-1} indicates presence of β linked glycosidic bond. Presence of 1,3—›1,6 linkage can be determined by presence of peak at and near the position 1040-1044 cm^{-1}, 1073-1079 cm^{-1}, 1160 cm^{-1} and 1200 cm^{-1}. The absence of peak

near and at these position indicates absence of 1—›6 linkage. The absence of 1—›6 linkage was found in FSSC isolates, CSH1 and Cc50, FDSC isolate Cc119. Spectra of extracted glucan from all pathogenic isolates was very similar to spectra of standard glucan. Table 4.4 shows the IR absorption frequency and corresponding functional group and linkages.

Table 4.4: Functional group and their corresponding absorption frequencies in FTIR spectra of β-glucan.

Functional Group	Frequency range (cm^{-1})
-OH Group	3400-3451
-C-H bond	2915-2940
-C=O bond	1612-1644, 1748, 1378
Polysaccharide	950-1200
1—›3 linkage	1156-1165
-C-O bond	1144-1162
-COO group	1318, 1420, 1450, 1616, 1627
β linked glycosidic bond	875-901
1—›6 linkage	1040-1044, 1073-1079, 1160, 1200

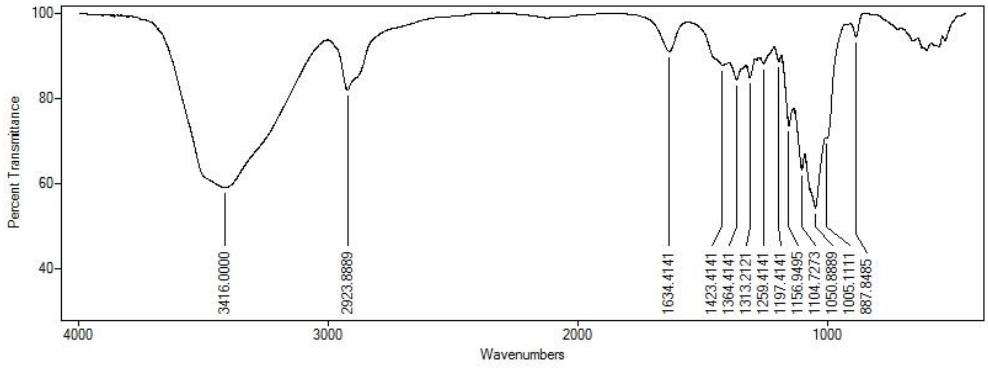

Figure 4.3: FTIR spectra of Standard β-1,3-glucan from *E. gracilaris*.

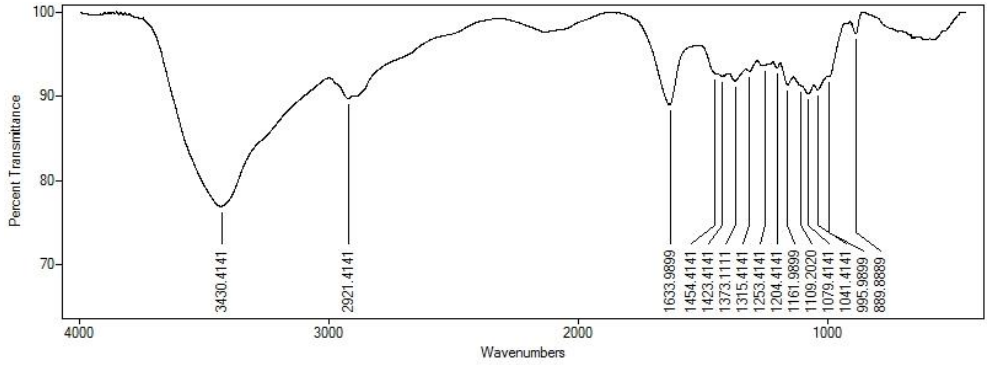

Figure 4.4: FTIR spectra of Standard Curdlan.

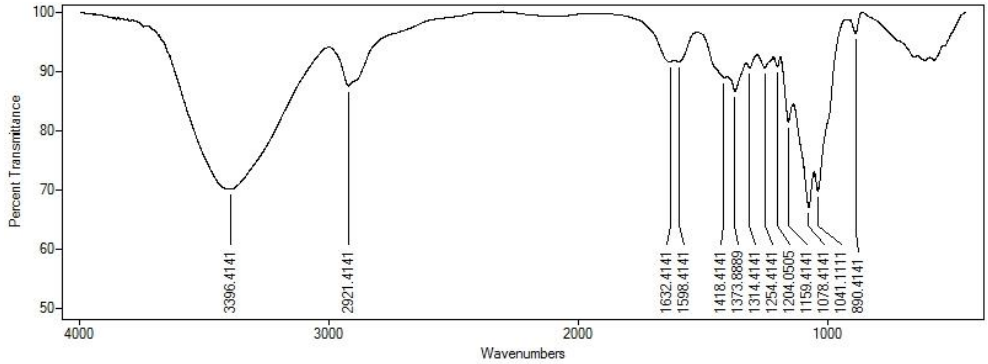

Figure 4.5: FTIR spectra of Standard Laminarin.

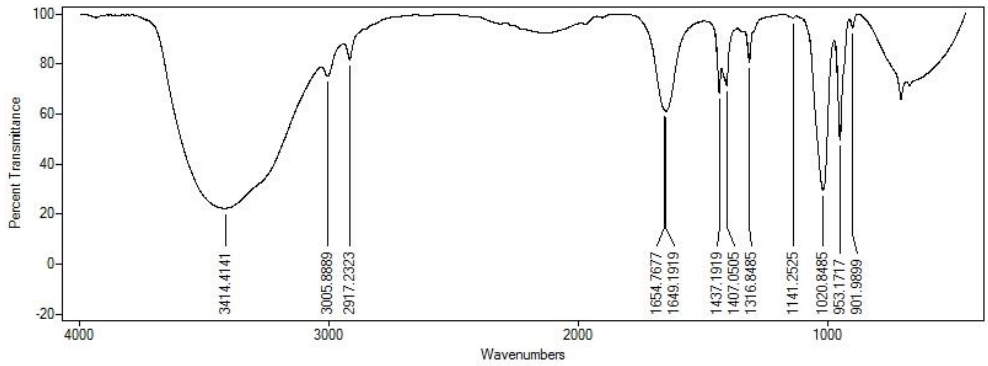

Figure 4.6: FTIR spectra of crude β-glucan extracted from FSSC CSH1.

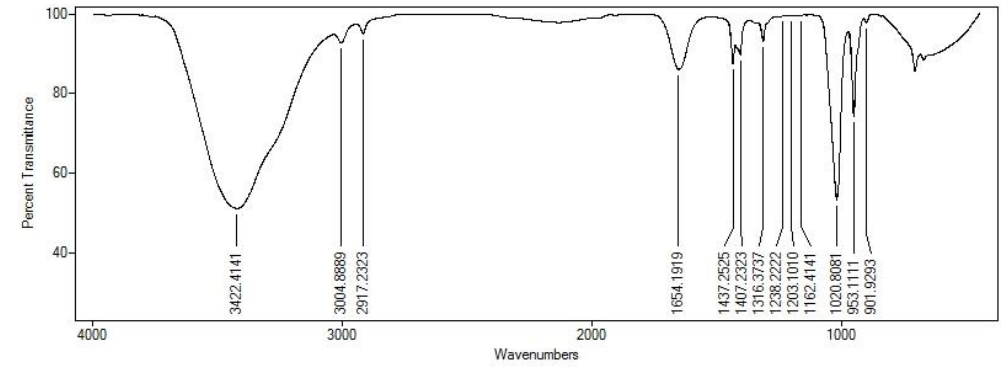

Figure 4.7: FTIR spectra of crude β-glucan extracted from FSSC CSH2.

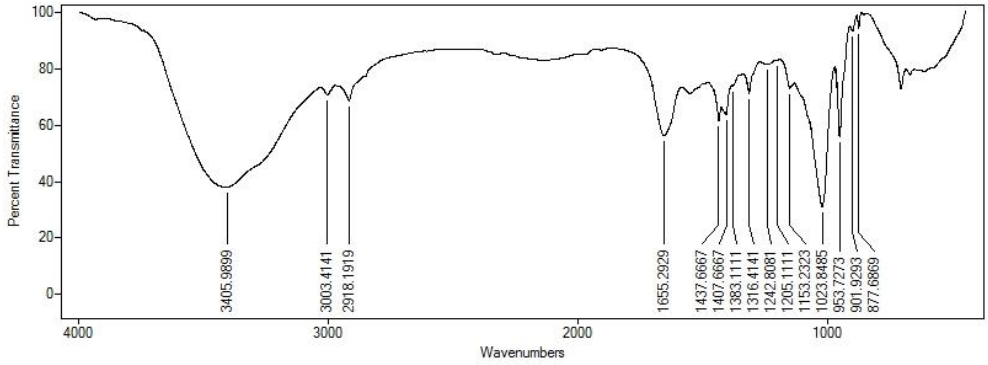

Figure 4.8: FTIR spectra of crude β-glucan extracted from FSSC CSH3.

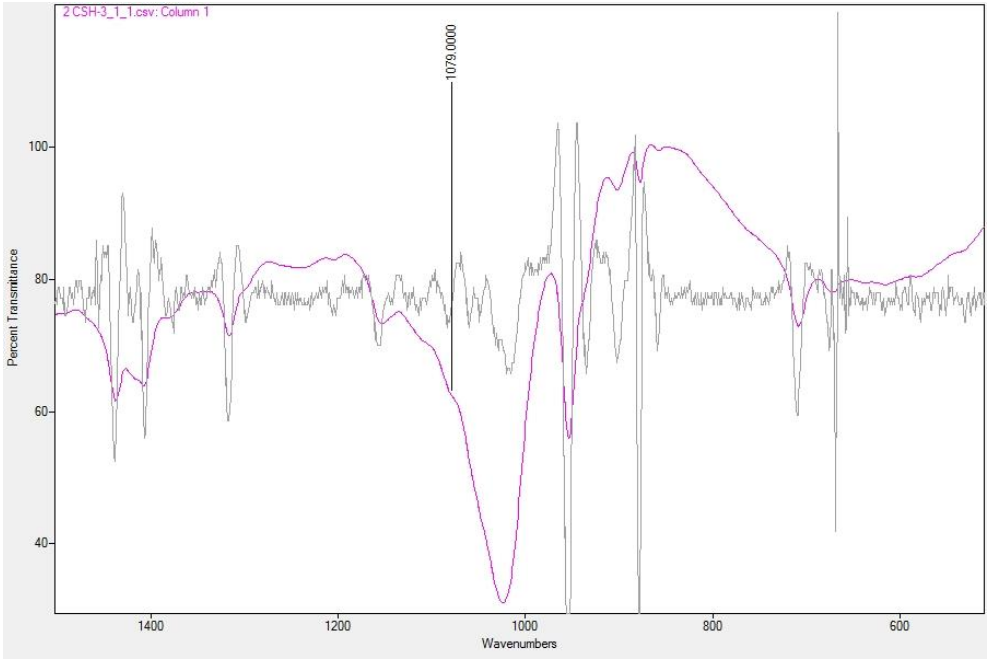

Figure 4.9: Zoomed FTIR spectra of crude β-glucan extracted from FSSC CSH3 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

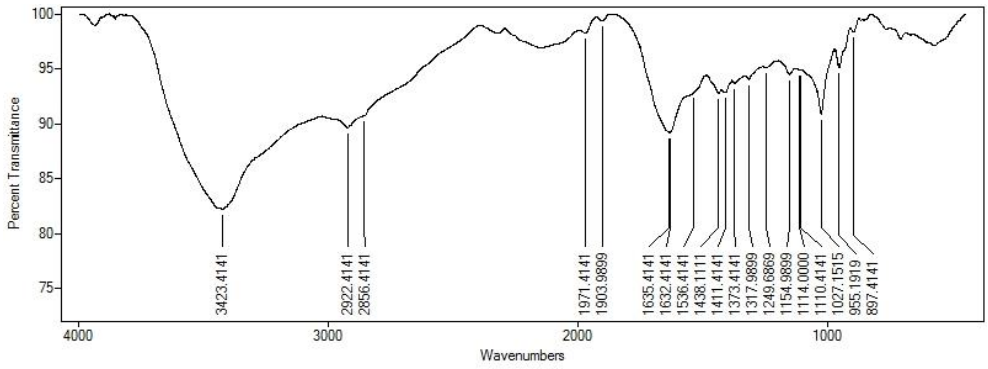

Figure 4.10: FTIR spectra of crude β-glucan extracted from FSSC CSH5.

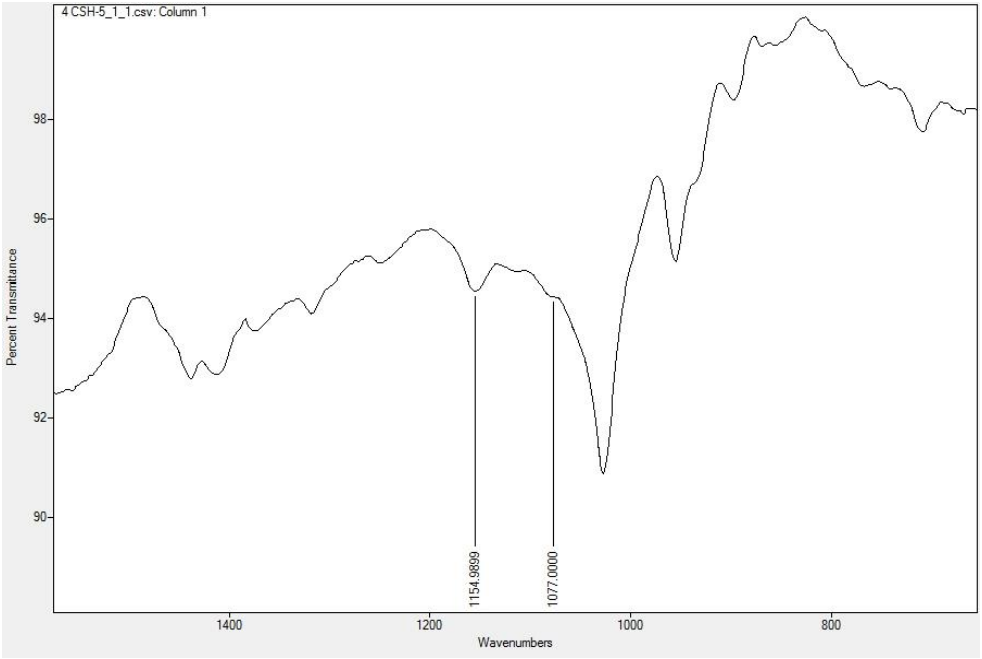

Figure 4.11: Zoomed FTIR spectra of crude β-glucan extracted from FSSC CSH5 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

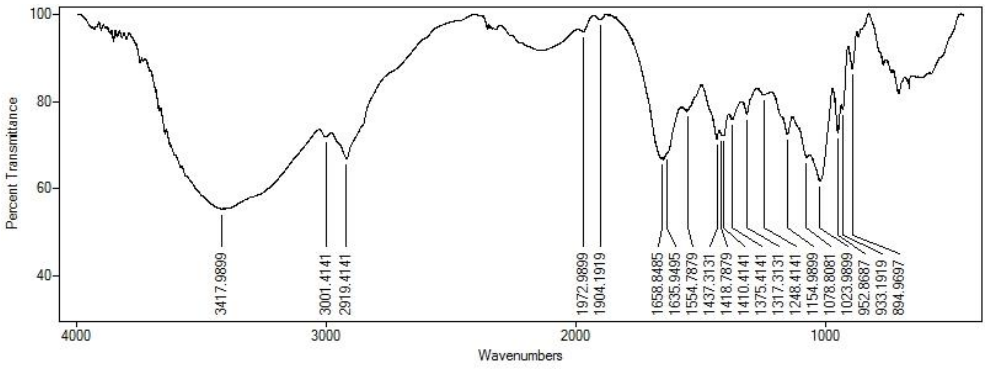

Figure 4.12: FTIR spectra of crude β-glucan extracted from FSSC CSH6.

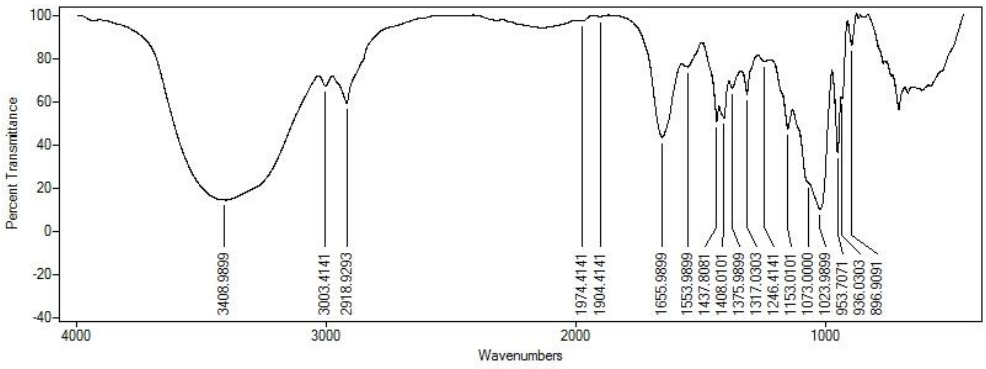

Figure 4.13: FTIR spectra of crude β-glucan extracted from FSSC CSH7.

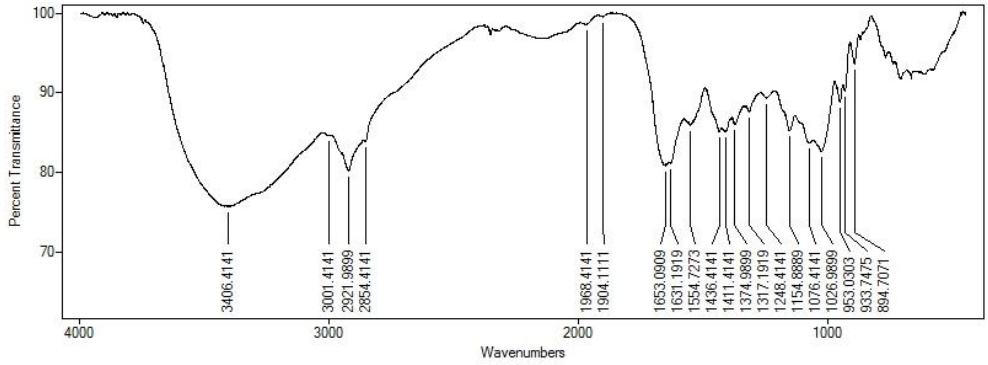

Figure 4.14: FTIR spectra of crude β-glucan extracted from FSSC CSH8.

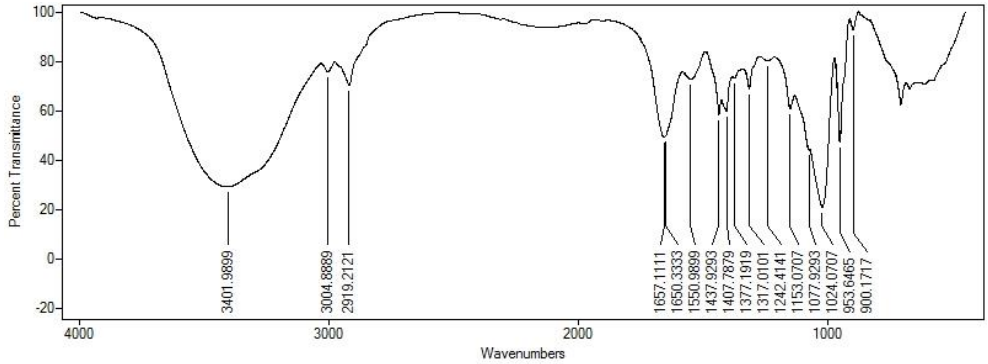

Figure 4.15: FTIR spectra of crude β-glucan extracted from FSSC CSH9.

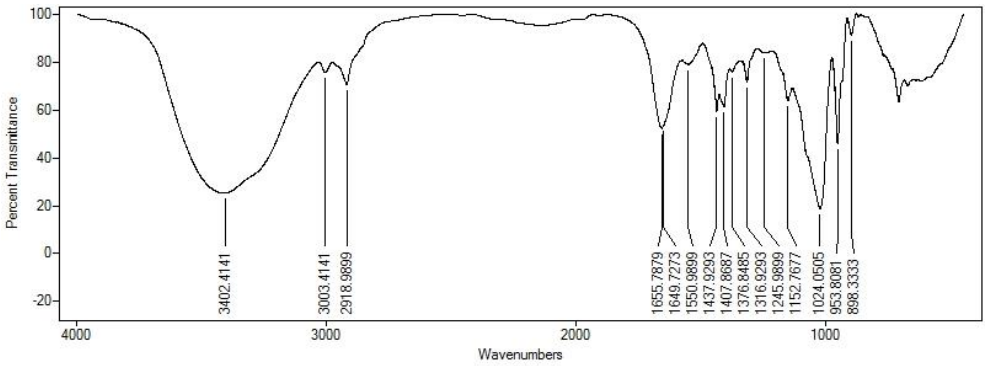

Figure 4.16: FTIR spectra of crude β-glucan extracted from FSSC CSH10.

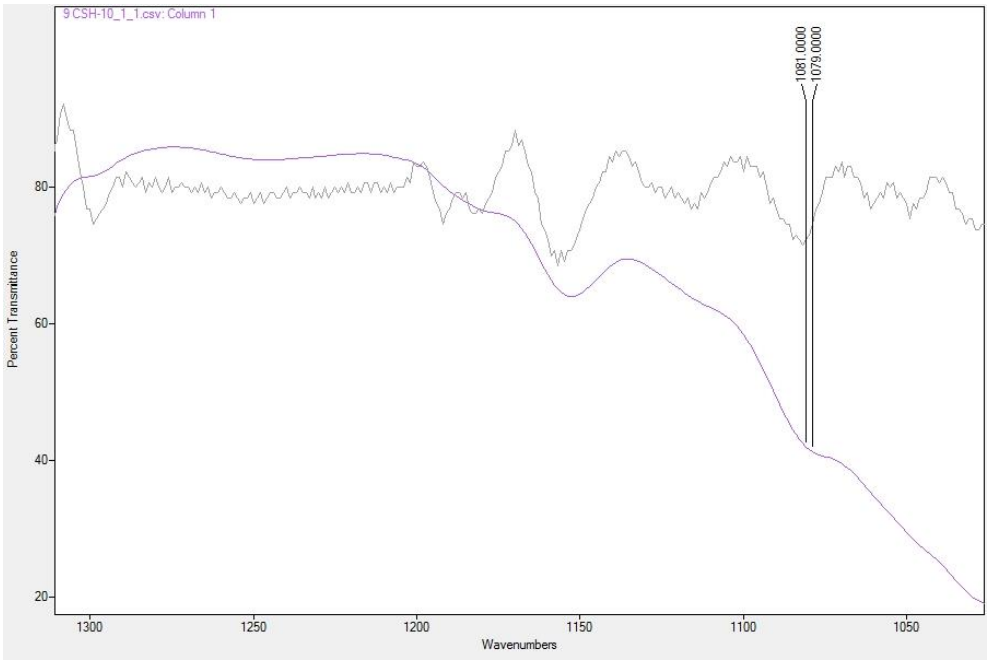

Figure 4.17: Zoomed FTIR spectra of crude β-glucan extracted from FSSC CSH10 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

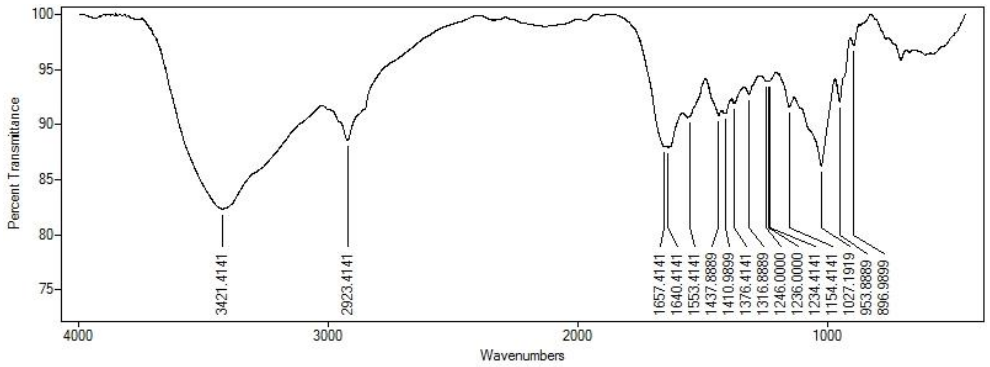

Figure 4.18: FTIR spectra of crude β-glucan extracted from FSSC CSH11.

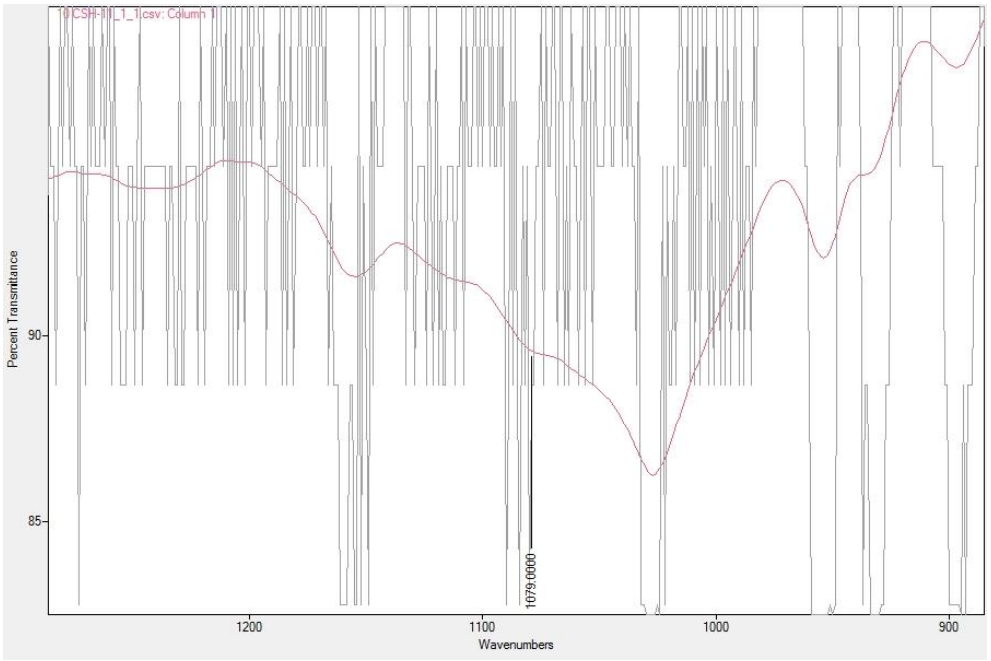

Figure 4.19: Zoomed FTIR spectra of crude β-glucan extracted from FSSC CSH11 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

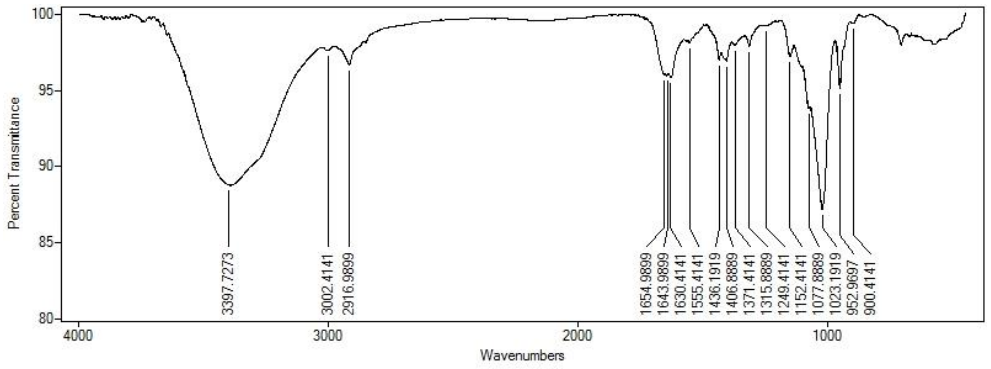

Figure 4.20: FTIR spectra of crude β-glucan extracted from FSSC Cs1.

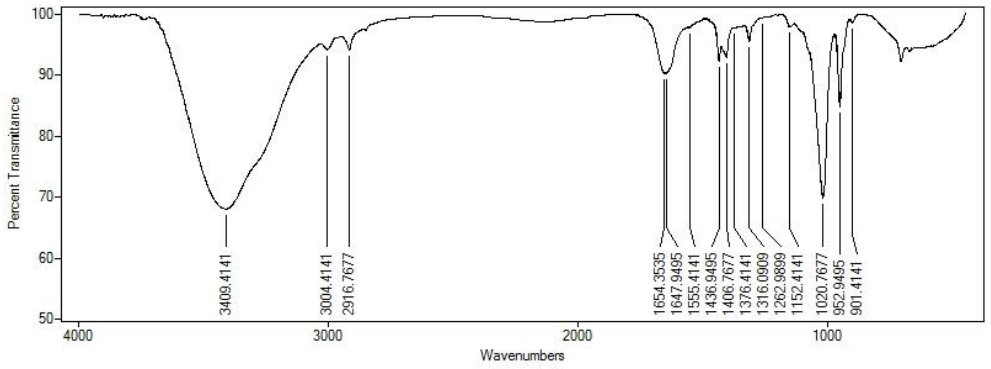

Figure 4.21: FTIR spectra of crude β-glucan extracted from FSSC Cs2.

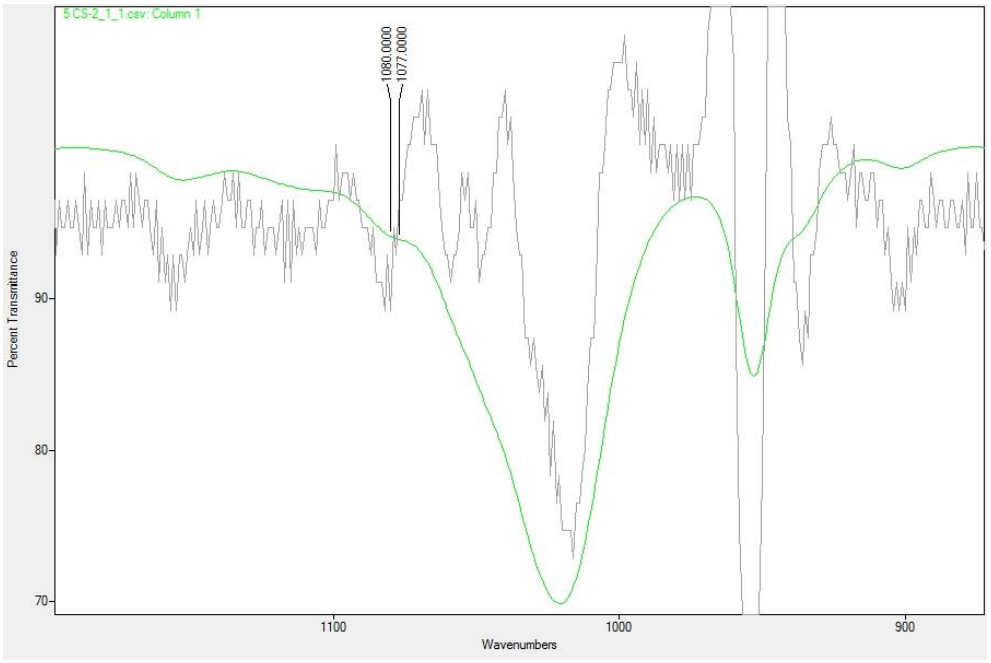

Figure 4.22: Zoomed FTIR spectra of crude β-glucan extracted from FSSC Cs2 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

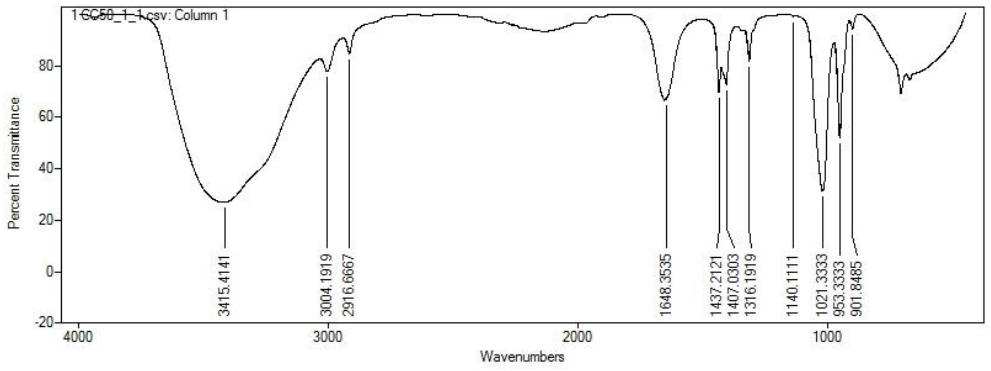

Figure 4.23: FTIR spectra of crude β-glucan extracted from FSSC Cc50.

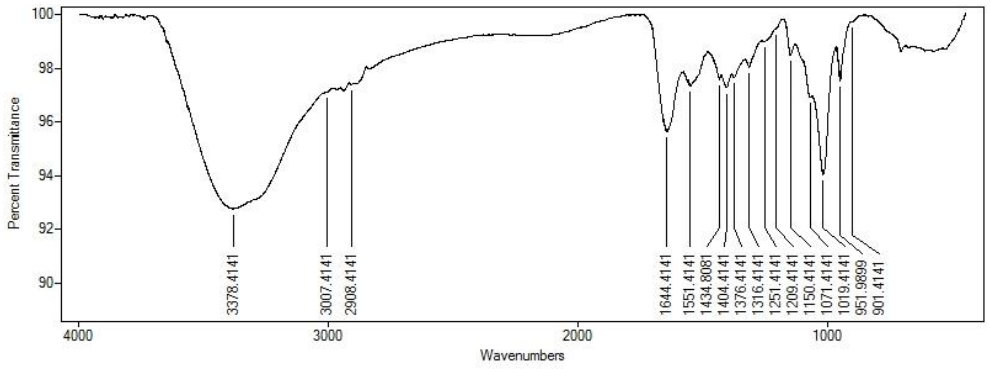

Figure 4.24: FTIR spectra of crude β-glucan extracted from FSSC Cc240.

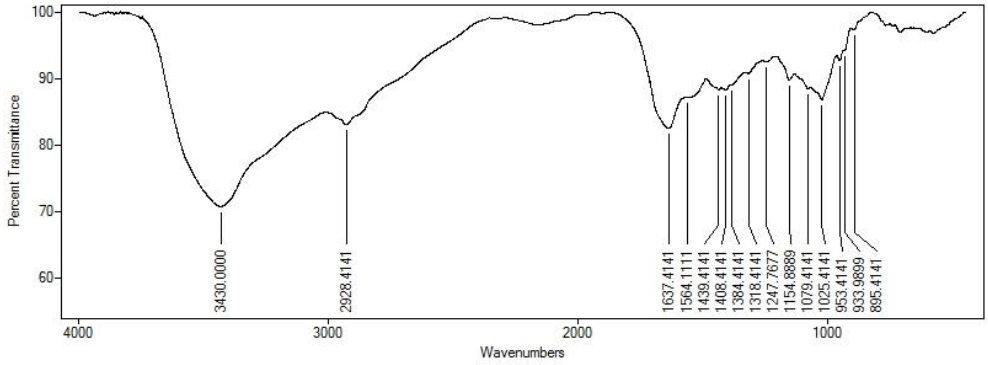

Figure 4.25: FTIR spectra of crude β-glucan extracted from FDSC Cc26.

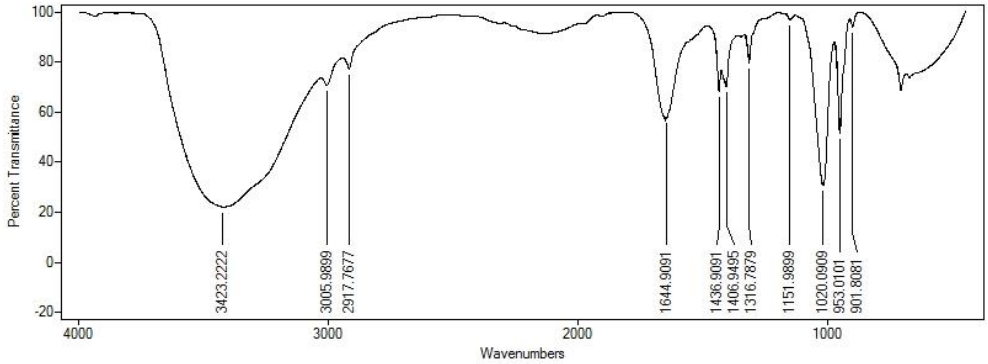

Figure 4.26: FTIR spectra of crude β-glucan extracted from FDSC Cc119.

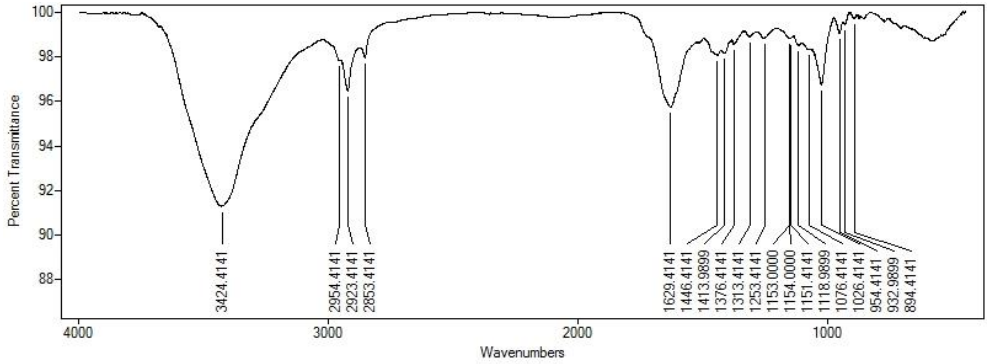

Figure 4.27: FTIR spectra of crude β-glucan extracted from FDSC CSH4.

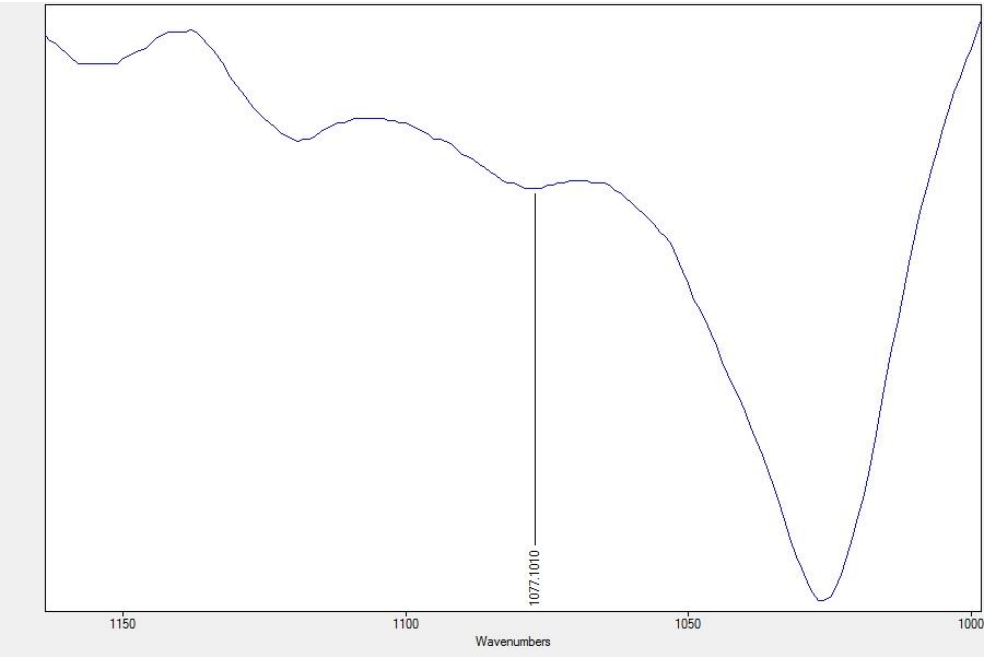

Figure 4.28: Zoomed FTIR spectra of crude β-glucan extracted from FDSC CSH4 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

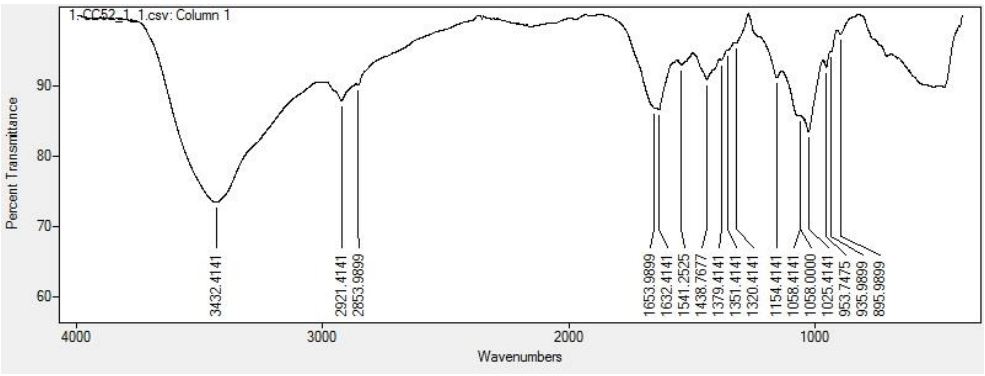

Figure 4.29: FTIR spectra of crude β-glucan extracted from *F.sacchari* Cc52.

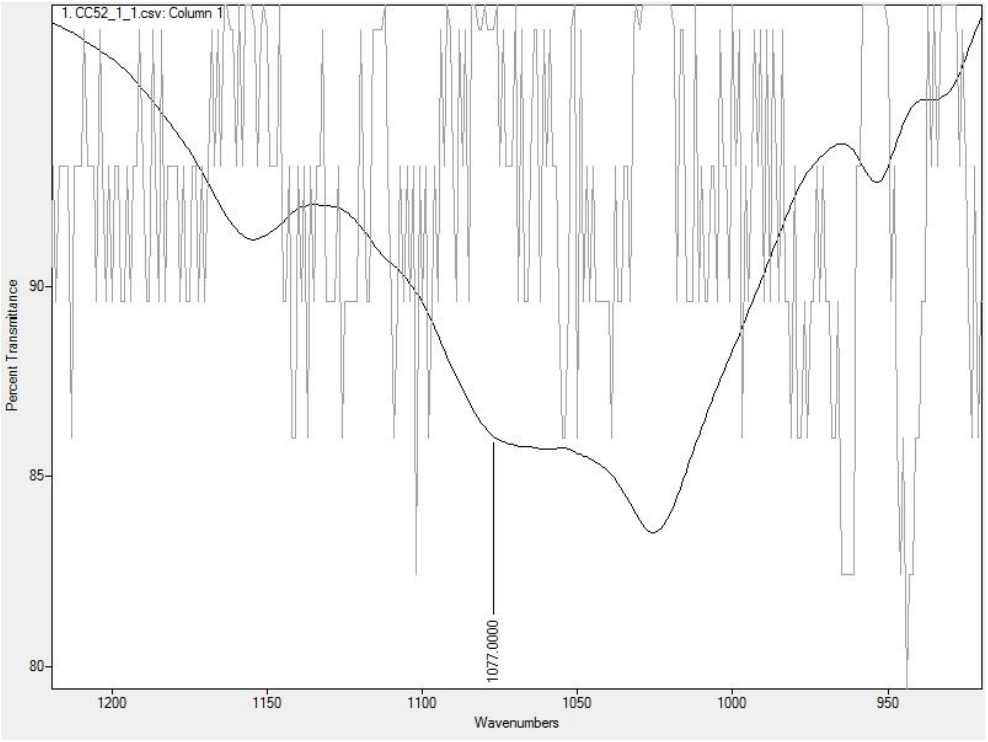

Figure 4.30: Zoomed FTIR spectra of crude β-glucan extracted from *F.sacchari* Cc52 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

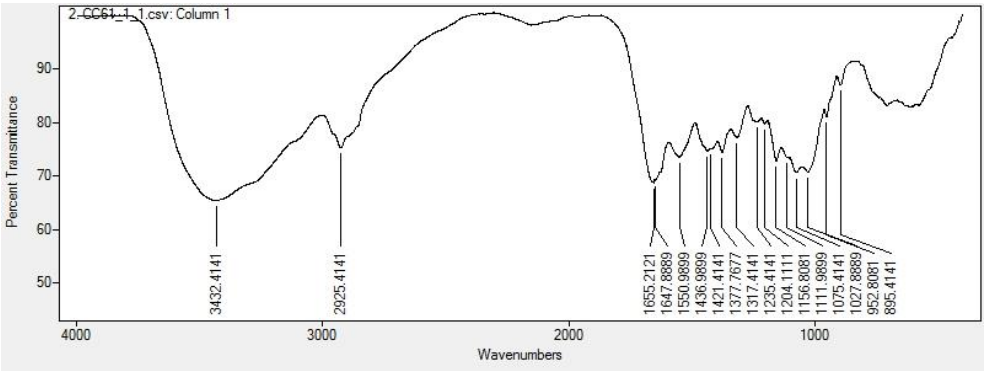

Figure 4.31: FTIR spectra of crude β-glucan extracted from *F.sacchari* Cc61.

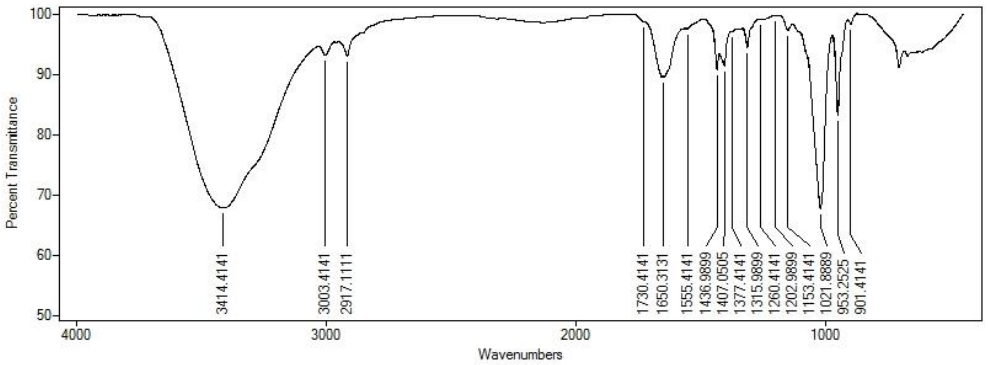

Figure 4.32: FTIR spectra of crude β-glucan extracted from *F.sacchari* Cc167.

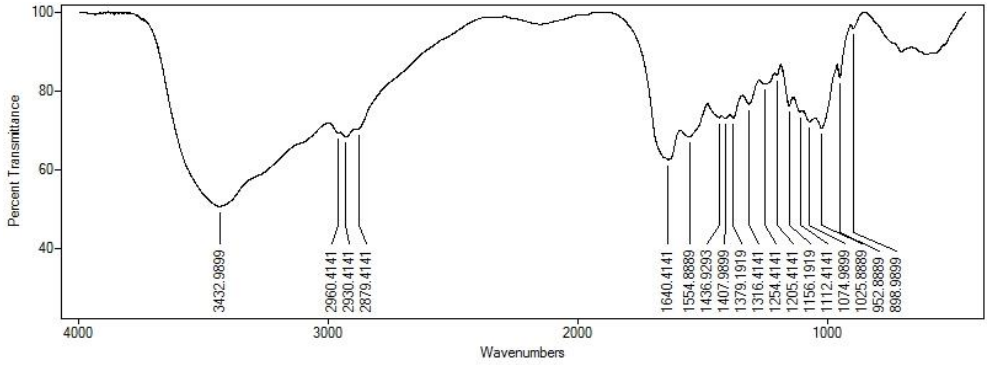

Figure 4.33: FTIR spectra of crude β-glucan extracted from *F.sacchari* Cc215.

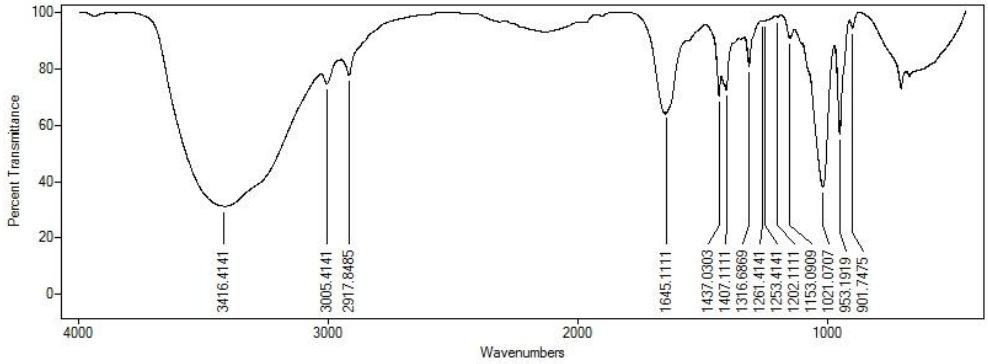

Figure 4.34: FTIR spectra of crude β-glucan extracted from *F. incarnatum-equiseti* Cc172.

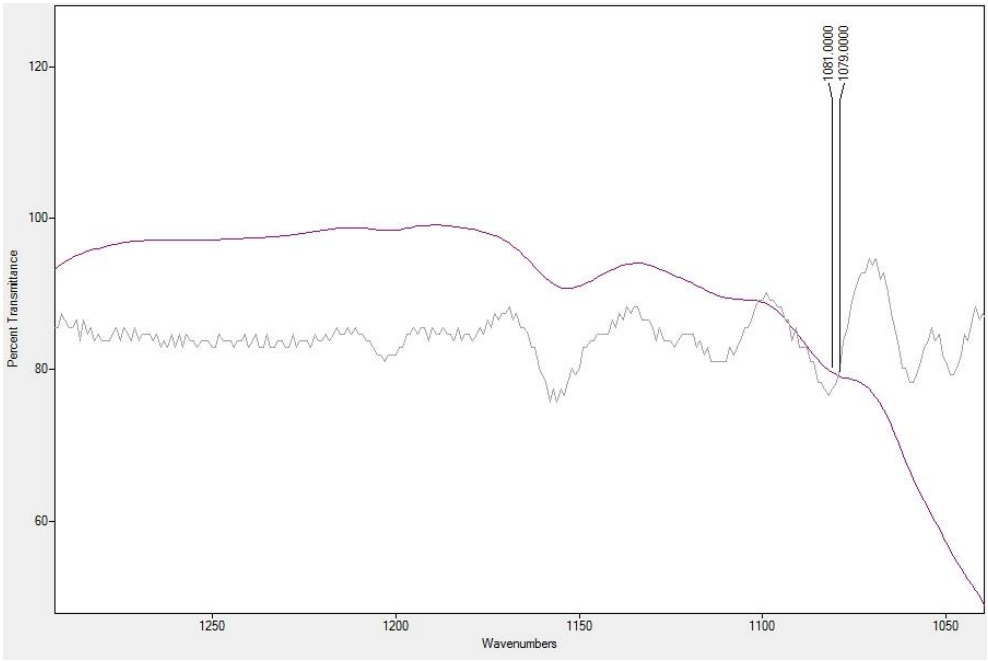

Figure 4.35: Zoomed FTIR spectra of crude β-glucan extracted from *F. incarnatum-equiseti* Cc172 to detect presence of 1—›6 linkage at 1073-1079 cm^{-1}.

4.3.5 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 and NCBI was used to find out Glucanases and Glucan synthase genes present in *N. haematococca*. Total 8 genes were found in *N. haematococca* and primers were designed agained them (Table 4.1). Six genes belonged to Glucanses (G1-G6) and two belonged to Glucan synthase (FKS1 and GS2). Among the eight genes, only 2 were characterized in literature: FKS1 as glucan synthase (Glycosyl Hydrolase Family) and Glucanase 4 as Glycoside Hydrolase. Others were hypothetical proteins and yet to be annotated and characterized. Figure 4.36 to Figure 4.38 shows relative expression of glucanases (G1, G3, G5, G6) and glucan synthases (FKS1, GS2) in CSH1, CSH2, CSH3, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1, Cs2, Cc50 and Cc240. Expression of G3, FKS1 and GS2 was high in maximum isolates. In all FSSC isolates, G2 and G4 were not getting expressed. Both Glucan synthase genes (FKS1 and GS2) showed expression in all isolates; while G3 was the only glucanase showing expression in maximum number of isolates. Differences in gene expression were found in some isolates and for some genes. In Cs1, Cs2 and Cc50, GS2 was not expressed. In Cc50 expression of G3 was also not found. The relative gene expression of G3 was higher in CSH1, CSH2, CSH5, CSH7, CSH8, CSH9, CSH10 and Cc240. The relative gene expression of FKS1 was higher in CSH1, CSH2, CSH5, CSH7, CSH8 and Cc240. The relative gene expression of GS2 was higher in CSH1, CSH2, CSH3, CSH5, CSH11 and Cc240.

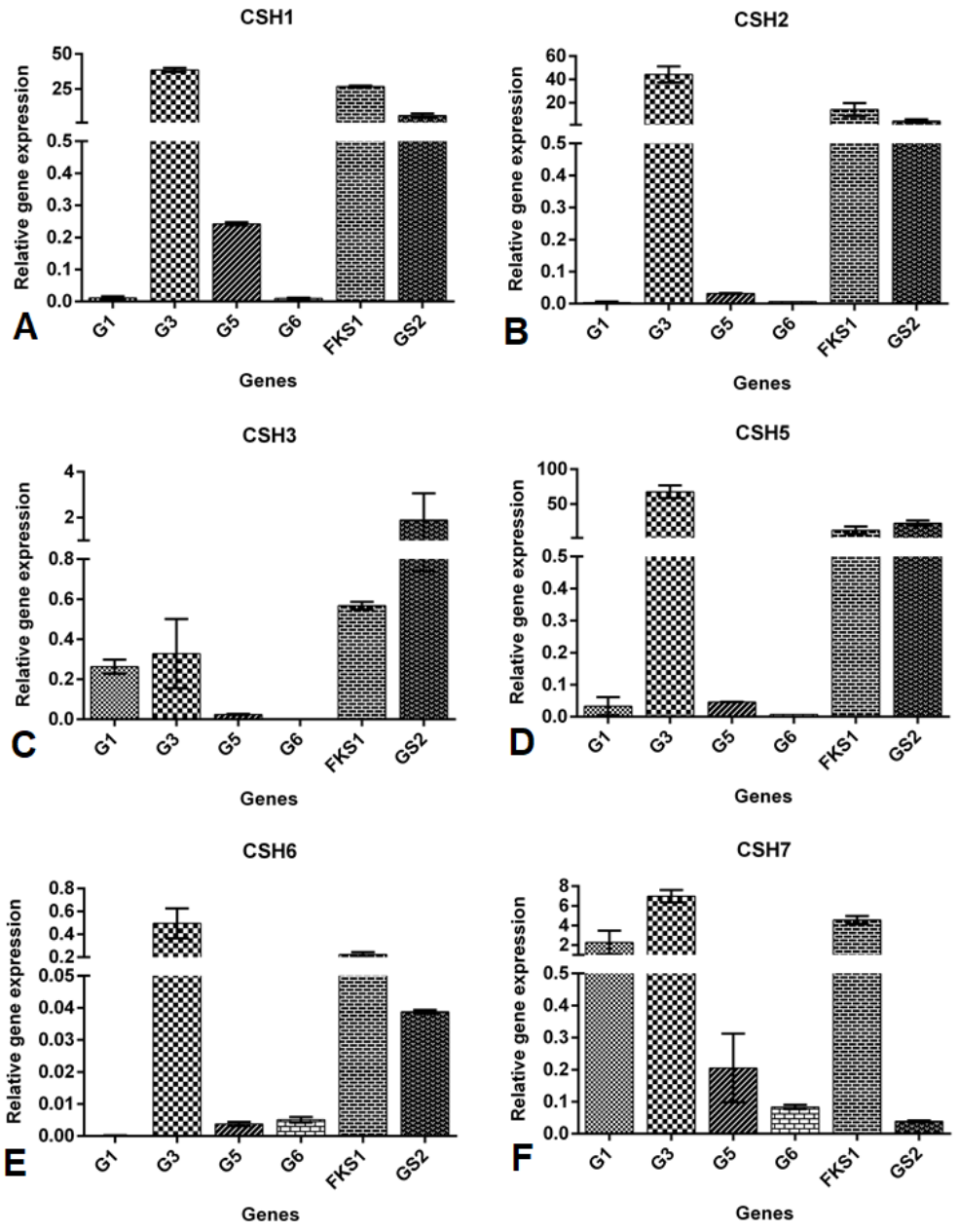

Figure 4.36: Relative gene expression of G1, G3, G5, G6, FKS1 and GS2 in FSSC isolates. (**A)** FSSC CSH1, **(B)** FSSC CSH2, **(C)** FSSC CSH3, **(D)** FSSC CSH5, **(E)** FSSC CSH6 and **(F)** FSSC CSH7. Expression of G3, FKS1 and GS2 was highest in CSH1, CSH2, CSH5 and CSH6. In FSSC CSH3 GS2 was highly expressed. Expression of G1 was higher in CSH7. Statistically significant difference was considered at $p<0.05$.

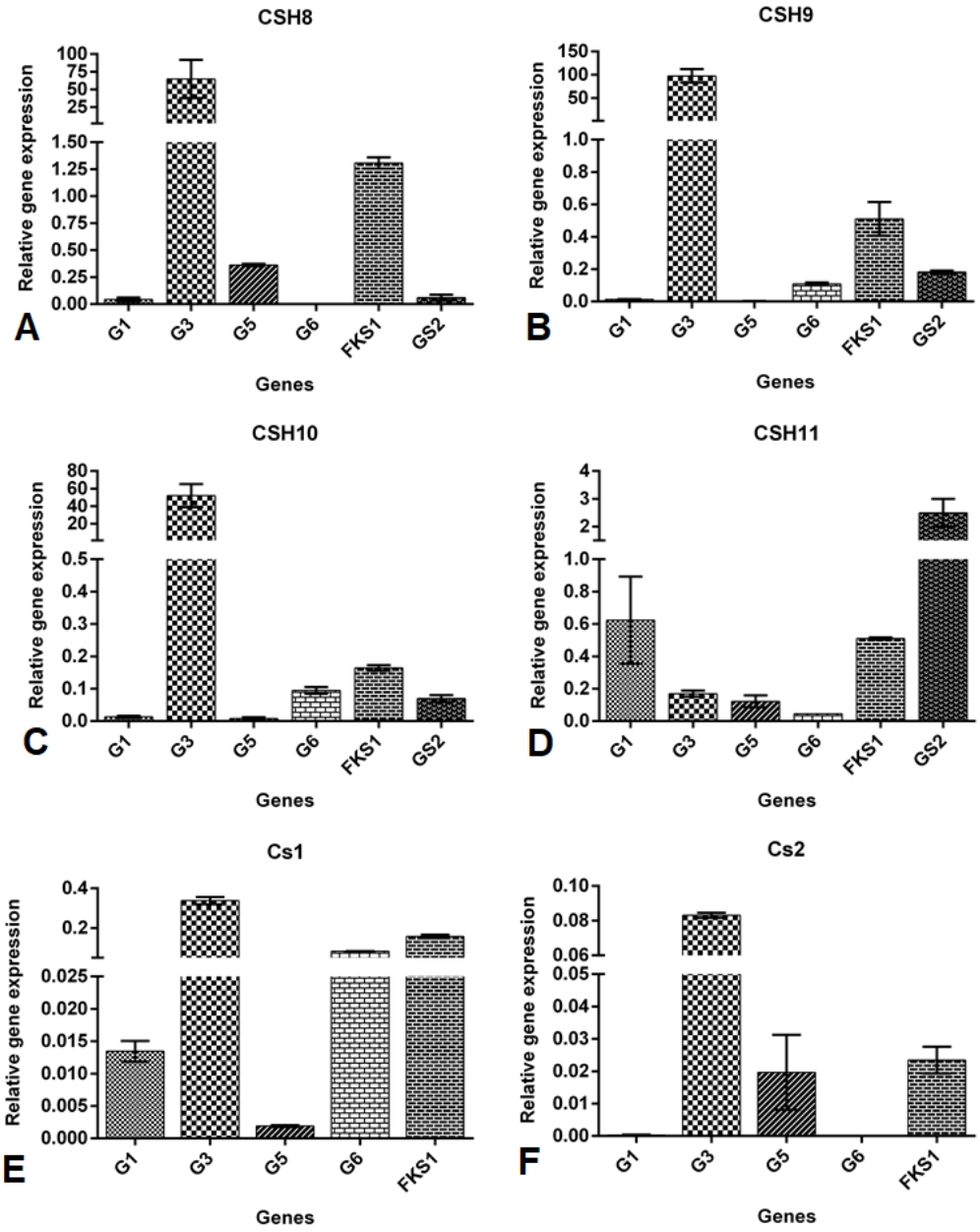

Figure 4.37: Relative gene expression of G1, G3, G5, G6, FKS1 and GS2 in FSSC isolates. (**A)** FSSC CSH8, **(B)** FSSC CSH9, **(C)** FSSC CSH10, **(D)** FSSC CSH11, **(E)** FSSC Cs1 and **(F)** FSSC Cs2. Expression of G3 was high in all isolates except CSH11. GS2 was not expressed in Cs1 and Cs2. Statistically significant difference was considered at $p<0.05$.

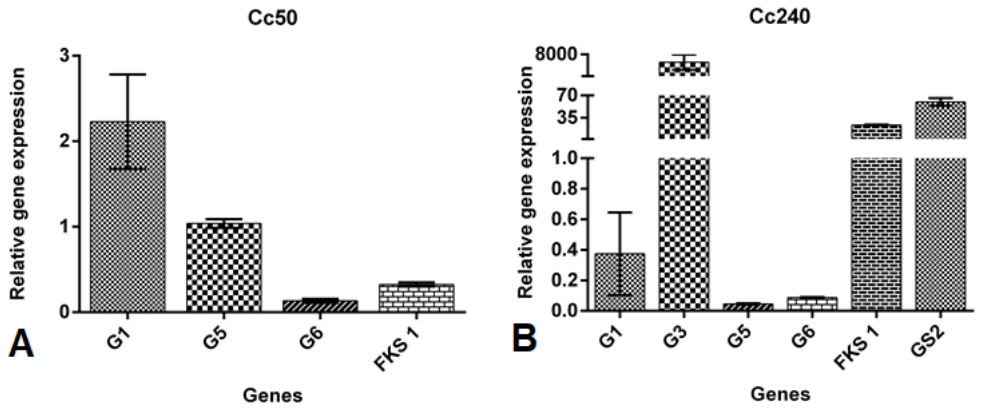

Figure 4.38: Relative gene expression of G1, G3, G5, G6, FKS1 and GS2 in FSSC isolates. (**A)** FSSC Cc50 and **(B)** FSSC Cc240. G3 was expressed in Cc240 but was not expressed in Cc50, also GS2 was not expressed in Cc50. FKS1 and GS2 both are highly expressed in Cc240. Statistically significant difference was considered at $p<0.05$.

4.3.6 Immunomodulatory effect of β-glucan from *Fusarium* spp. on human whole blood:

Whole human blood was used to study the immunomodulatory effects of standard β-glucan (curdlan and laminarin) and fungal mycelia by estimating TNF alpha production (Figure 4.39). The TNF alpha was estimated using ELISA method with the help of human TNF alpha kit. Curdlan (β-1,3-glucan) induced more TNF alpha production compared to laminarin (β-1,3-1,6-glucan). The immunomodulatory effects of the fungal isolates (CSH4 and CSH5) were evaluated using; (1) extracted beta glucan, (2) fragmented mycelia and (3) NaOH treated mycelia (to expose beta glucan). Extracted β-glucan from FDSC CSH4 induced more TNF alpha production. Mycelia of FDSC CSH4 and FSSC CSH5 induced similar TNF alpha production. NaOH treated Mycelia of FDSC CSH4 induced more TNF alpha production compared to NaOH treated FSSC CSH5 mycelia. The differences in TNF alpha production when treated with extracted β-glucan indicate the differences in their fibrillar structure. The higher TNF alpha production upon induction with NaOH treated CSH4 indirectly indicate the presence of higher β-glucan in CSH4.

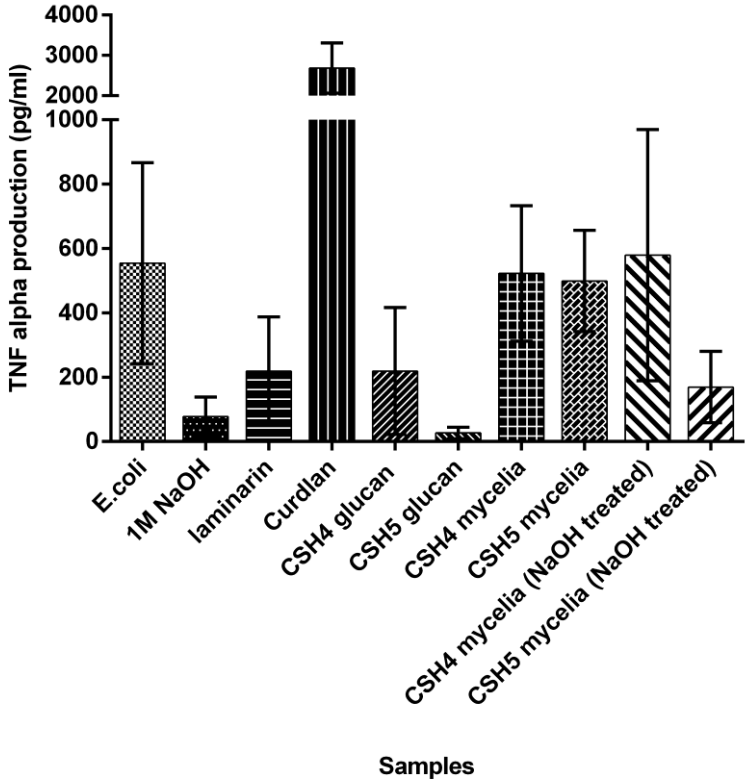

Figure 4.39: TNF alpha estimation using ELISA in whole blood cells to study the effect of β-glucan. Curdlan induced highest TNF alpha production. FDSC CSH4 β-glucan and mycelia after NaOH treatment induced more TNF alpha production. Statistically significant difference was considered at $p>0.05$.

4.4 Discussion:

β-glucan is major structural polysaccharide of fungal cell wall, constituting approximately 50–60% of the wall by dry weight. In yeast and filamentous fungi β-1,3-glucan constitutes 65 and 90% of cell wall mass (Bowman and Free, 2006). During cell wall synthesis, plasma membrane associated glucan synthase and chitin synthase complexes create chitin UDP-N-acetylglucosamine and β-1,3-glucan chains by incorporating UDP-glucose molecules. These linear chains can pass in to and out of cell wall space (Beauvais *et al*., 1993; Fesel and Zuccaro, 2016). β-glucan is most abundant and highly variable structure of fungal cell wall. The β-1,3-glucan chains are connected via 1—›6 linkage to form β-1,3-1,6-glucan (Fesel and Zuccaro, 2016).

Several methods has been reported for β-glucan extraction from grains, yeast and filamentous fungi. Extraction of β-glucan generally involves chemical, physical and enzymatic processes. Chemically, fungal cells can be lysed by NaOH, HCl, acetic acid, citric acid and using other chemically aggressive solutions and in most cases chemical lysis is held at higher temperatures. Physically fungi are disrupted using sonication, homogenizers with high pressure.

Extraction of *S. cerevisiae* β-glucan using alkali hydrolysis followed by enzymatic treatment has been reported. The β-glucan was recovered as insoluble pellet quantified using borosulfuric acid method and HPLC method. (Boone *et al*., 1990; Hong *et al*., 1994). β-glucan has been extracted using mechanical methods also. β-glucan extraction from *S. cerevisiae* has been carried out with glass beads to break the cells followed by alkali extraction and enzymatic treatment (Umeyama *et al*., 2006). β-glucan can be extracted from cereal seeds or products using NaOH, Na_2CO_3 and using hot water with thermostable α-amylase (Rimsten *et al*., 2002). Beer and group has reported use of 25 times less NaOH for extraction of β-glucan than the amount reported by Rimsten and group. Higher amount of NaOH can be related to degradation of β-glucan and yield a low molecular weight β-glucan (Beer *et al*., 1997). In one report β-glucan extraction from barley has been carried out using NaOH and neutrilised with HCl. The β-glucan precipitation was carried out with 80% ethanol followed by centrifugation to obtain pellet of β-glucan and estimation was carried out with ELISA using antibody specific for β-glucan (Rampitsch *et al*., 2003). Glucan enzymatic method (GEM) assay has also reported for β-glucan extraction. In GEM assay, glucan is extracted using sodium acetate and lyticase solution (Tris, EDTA, NaCl in KOH solution) followed by enzymatic digestion (Ciucanu, 2006). Sodium hypochloride extraction and enzymatic digestion (SEED) assay has been developed for β-glucan extraction. The β-glucan is extracted from barley using sodium hypochloride, DMSO and NaOH followed by enzymatic digestion and quantification carried out using glucoseoxidase/peroxidase (GOPOD) assay spectrophotometrically at 510 nm (Ide *et al*., 2018). β-glucan quantification from various mushrooms and yeast had been carried out. One method is fluorescence microassay using aniline

blue, a fluorescent dye. Aniline blue binds to β-1,3-glucan in the cell wall (Ko and Lin, 2004; Rieder *et al*., 2018). Several tests have been developed to estimate β-glucan level in patients suffering from systemic fungal diseases like, candidiasis, aspergillosis, fusariosis, pneumocystis and many more. These tests were also used to quantitate β-glucan level from yeast, fungi and mushrooms. These tests includes, Limulus amebocyte lysate (LAL) method (Johnson *et al*., 2010), the modified LAL, Glucatell assay (Cherid *et al*., 2011), Fungitell assay and wako β-glucan test (Friedrich *et al*., 2018). McCleary and Darga had reported comparison of hydrochloric acid, sulphuric acid and trifluoroacetic acid (TFA) extraction method of β-glucan extraction from mushroom. The yield of β-glucan was higher in sulphuric acid and hydrochloric extraction compared to TFA extraction (McCleary and Draga, 2016). In present study we have used NaOCl-DMSO method (Ohno *et al*. 1999) and NaOH-acetic acid method (Williams *et al*. 1991 and Ohno *et al*. 1999) for extraction of β-glucan. The maximum amount of soluble β-glucan was obtained using NaOCl-DMSO method and it was soluble in DMSO and was capable of further estimation.

β-glucan estimation has been reported from several grains and mushrooms. β-glucan quantification from shiitake mushroom was carried out using magzyme assay kit, K-YBGL. The amount of β-glucan was found to be 2.87±0.09 g/100gm of dry weight (Zrnić-Ćirić *et al*., 2019). Magzyme assay kit has also been used to estimate the β-glucan level in yeast powder and mushroom (Apostu *et al*., 2017; Sari *et al*., 2017). Berley glucan, *Ganoderma lucidum* and *Sarcomyxa serotine* β-glucan quantification using GOPOD assay yielded 92.4±5.08 %, 30.0±1.05 % and 19.6±0.39 %, respectively. In GOPOD test, the GOPOD reagent is mixed with provided glucose oxidase or peroxide. 160 µl of this mixture is added to 40 µl samples in 96 well plate and incubated at 37˚C for 20 minutes and absorbance is measured spectrophotometrically at 510 nm (Ide *et al*., 2018).

In candidiasis patients the β-glucan levels estimated with fungitell and wako β-glucan test, median β-glucan levels were 351pg/ml and 8.4pg/ml, respectively. The fungitell test was considered superior to wako β-glucan test

(Friedrich *et al*., 2018). β-glucan content in yeast cells was treated with Glucanex® 200G contains mainly β-1,3-glucanase and some β-1,6-glucanase. The releases glucose was estimated using HPLC. The yield of β-glucan in *C. bombicola, C. albicans* and *S. cerevisiae* was 0.08gm/gm, 0.025gm/gm and 0.03gm/gm of dried cells, respectively (Kim *et al*., 2004). Quantification of β-glucan from tear film of mycotic keratitis and bacterial keratitis patients were carried out using Fungitech G test. The β-glucan level before and after corneal scrapping from mycotic keratitis patients were 184±128 pg/ml and >1000 pg/ml, respectively. The β-glucan level before and after corneal scrapping from bacterial keratitis patients were 5.8±2.6 pg/ml and 8.2±5.2 pg/ml, respectively (Kaji *et al*., 2009). β-glucan quantification from *Sporothrix schenckii* and *S. brasiliensis* was carried out using hexose estimation using anthrone method (anthrone in H2So4), the yield was 25.40±1.18% and 17.55±0.55% (Lopes-Bezerra *et al*., 2018).

In present study, the yield of β-glucan obtained from clinical isolates of *Fusarium* spp. is in wide range. β-glucan concentration from other pathogenic fungal spp. has been reported. *Pythium insidio-sum* contains ≈ 20% β-glucan ,*Aspergillus candidus* contains ≈ 3.4% β-glucan, *F. graminearum* and *Trichoderma harzianum* contains ≈ 6% β-glucan, *Penicillium* and *Paecilomycesspp* contains ≈ 17-23% β-glucan of dried mycelium (Kyanko *et al*., 2013). The total glucan contents was measured in *P. insidiosum*. The screening of the 13 *P. insidiosum* clinical isolates revealed that glucan contents ranging from 17.14 to 28.71 (% w/w) and β-glucan content ranging from 14.14 to 23.90 (% w/w). *P. aphanidermatum* had total glucan content of 24.90 (% w/w) and β-glucan content of 22.18 (% w/w) (Tondolo *et al*., 2017). In our study, we also found variation in glucan content in clinical isolates of *Fusarium* spp. and even within same species. Among 14 FSSC isolates it ranges from 4.0-12.5 (% w/w), in 3 FDSC isolates glucan content ranges from 7.8-80 (% w/w) and in 4 *F.sacchari* isolates it ranges from 4.2 - 81.5 (% w/w). Such high variation in the amounts of β-glucan within the same species is currently inexplainable and incomprehensible. These results warrant further detailed studies on the cell wall architecture of FDSC and *F. sacchari.*

In the present work estimation of β-1,3-glucan and total β-glucan was done using ELISA and Congo red assay (Mölleken *et al*., 2011), respectively. Antibodies used were specific for β-1,3-glucan. Congo red assay is based on the principle that an incorporation of Congo red dye into the triple helix leads to a bathocromic shift. The absorption maximum is moved from 493 to 523 nm. Therefore, Congo red can be used for characterization of glucan tertiary structures and it does not react with other polysaccharides (Guo *et al*., 2019; Villares, 2013; Wang *et al*., 2014; Yu *et al*., 2010). Some reports are there of using fluorescence aniline blue assay along with congo red assay to estimate β-1,3-glucan with β-1,3-1,6-glucan from mashroom. Yield of β-1,3-1,6-glucan ranges from 0.41- 3.28 g/100 g in *Pleurotus pulmonarius* and *Trametes versicolor* respectively. While β-1,3-glucan yield vary between 2.53-4.94 g/100g in *P. pulmonarius and Grifola frondosa* mycelia respectively (Nitschke *et al*., 2011). Enzyme inhibition assay has been developed to estimate β-1,3-glucan from occupational environment, plant and house dust (Douwes *et al*., 1996). Mölleken and group has reported quantification of β-1,3-glucan using aniline blue assay, β-1,3-glucan concentration was ranging from 2.5 ± 0.1g/ 100g in *P. pulmonarius* to 6.8 ± 0.2g/100g in *T. versicolor* and β-1,3-1,6-glucan using congo red assay was ranging from 0.4 ±0.0 g/ 100g in *P. pulmonarius and* 4.0 ±0.2 g/ 100g in *M. esculenta* dried mycelium (Mölleken *et al*., 2011). ELISA assay was originally developed as a diagnostic assay for systemic fungemia, to assay 1,3-β-D-glucans from pathogenic fungi. Estimation of β-glucan levels from *Aspergillus* species had been reported. β-glucan levels from *A. flavus, A. niger* isolates (both isolated from stored urine samples) and *A. ochaceus* (isolated from air sample from home dust) were 190 ng/mg, 160 ng/mg and 57 ng/mg, respectively using ELISA assay (Milton *et al*., 2001). The concentration obtained in this study was relatively less than what obtained in our study. β-1,3-glucan and β-1,6-glucan quantification from *S. cerevisiae* was carried out using HPLC. Yield of β-1,3-glucan was 174.2±30.6 mg/gm and 168.9±23.9 mg/gm of dry weight. Yield of β-1,6-glucan was 21.9±4.0 mg/gm and 21.3±2.8 mg/gm of dry weight (Hong *et al*., 1994). In *C. albicans*, β-1,3-glucan and β-1,6-glucan estimation was carried out and the amount was ~200µg/mg and ~60µg/mg of dry weight, respectively (Umeyama *et al*., 2006). β-glucan quantification was carried out from *C.*

albicans, C. glabrata, C. tropicalis, C. dubliniensis, C. krusei and *C. parapsilosis* using mouse IgG antibody specific for β-1,3-glucan. The yield of β-glucan was ~320µg/mg, ~210µg/mg, ~150µg/mg, ~310µg/mg, ~110µg/mg and ~340µg/mg of dry weight, respectively (Sem *et al*., 2016).

Reports of quantification of β-glucan level in pathogenic *Fusarium* species are lacking. In the present study, fourteen isolates of FSSC, 3 isolates of *F. delphenoides*, 4 isolates of *F. sacchari* and 1 *F. incarnatum-equiseti* isolate were used. Among *all Fusarium isolates* β-1,3-glucan and total β-glucan concentration was consistent in FSSC isolates, while in *F. delphenoides* and *F. sacchari* there was huge disparity between isolates. These results further corroborate with our initial observations of high glucan levels in certain FDSC and *F sacchari* isolates and suggest further detailed studies on the cell wall architecture of these isolates.

Methods used for glucan characterization includes chromatographic techniques mainly TLC and HPLC and spectroscopic techniques mainly IR, NMR, Raman. H^1 NMR and C^{13} NMR are used to characterise β-glucan from *Pleurotus pulmonarius* (Smiderle *et al*., 2008), *Agaricus bisporus* and *A. brasiliensis* (Smiderle *et al*., 2013), *S. cerevisiae* (Du *et al*., 2012; Upadhyay *et al*., 2017), *Paenibacillus polymyxa* (Jung *et al*., 2007), *Sparassis crispa* (Tada *et al*., 2007). Standard β-glucan, laminaran, curdlan, yeast glucan and scleroglucan were characterised using two dimentional NMR (Kim *et al*., 2000).

FTIR is powerful technique for structural characterization of glucan. It is sensitive to anomeric configuration and position of glycosidic linkage. Two important region for polysaccharide structural characterization are 1,200-950 cm^{-1} called sugar region and 950-750 cm^{-1} called anomeric region. It is used to differentiate between different types of fungal glucan (Prado *et al*., 2005; Synytsya and Novak, 2014). FTIR has been used to characterise β-glucan from *Botryosphaeria* spp. (Barbosa *et al*., 2003), *Hericium erinaceus* (Lee *et al*., 2009), *Pleurotus ostreatus* (Synytsya and Novak, 2014), *Agaricus blazei, A. bisporus* (Gonzaga *et al*., 2013; Khan *et al*., 2015), *C. albicans* (Adt *et al*.,

2006), *S.cerevisiae* (Galichet *et al*., 2001; Pengkumsri *et al*., 2017), *A. terreus* (Costa *et al*., 2019).

In present study to characterize extracted crude β-glucan, primarily TLC was performed. Komaniecka & Choma (2003) have isolated and characterized periplasmic cyclic L-glucans of *Azorhizobium caulinodans* using TLC. The solvent system they have used for characterization was butanol: ethanol: water (5:5:4) V/V which yielded four spots on TLC plate after spraying with 20% H2SO4 (Komaniecka and Choma, 2003). In present study by using this solvent system, single spot was obtained on TLC after developing the plate with iodine vapour with R_f value of 8.3. Further structural characterization was done using FTIR analysis.

In the present study, FTIR analysis was done for extracted glucan from pathogenic *Fusarium* isolates,standard curdlan and laminarin. In the FTIR spectra broad and intense stretching at 3430.41 cm^{-1} as well as peak at 3419-3451 cm^{-1} is characteristics of hydroxyl group (Xia *et al*., 2014; You *et al*., 2014), the stretching at 2921.41-2923 cm^{-1} 2925 cm^{-1}, 2928-2940 cm^{-1} is attributed to C-H bond (Deng *et al*., 2012; Wang *et al*., 2005; Xia *et al*., 2014; You *et al*., 2014). The presence of polysaccharide in present study is depicted by occurrence of absorption band in region of 950-1200 cm^{-1} (Ahmad *et al*., 2010; Deng *et al*., 2012). The characteristic of –C=O bond is peak at 1612-1644 cm^{-1}, 1748 cm^{-1}, 1378 cm^{-1} (Wang *et al*., 2008, 2005; You *et al*., 2014). The band at 1156-1165 cm-1 corresponds to indicate linear structure of β-D-glucan linked through 1—›3 linkage and it is important to note that absorption peak at 875 cm-1 and 889-898 cm-1 indicate β linked glycosidic bond (Ahmad *et al*., 2010; Barbosa *et al*., 2003; Ding *et al*., 2010; Galichet *et al*., 2001; Xia *et al*., 2014). The peak at 1040-1044, 1073-1079, 1160, 1200 is characteristic of 1,6 linkage (Bzducha-Wróbel *et al*., 2019; Galichet *et al*., 2001; Howard *et al*., 1976; Synytsya and Novak, 2014). Glucan extracted from all the isolates were having closely similar spectra as standard. However in some of the isolates, the identification peak of β linkage was not seen in normal spectra. When the spectra was zoomed, in some of the isolates the peak was seen. From all *Fusarium* isolates, isolates which do not have 1—›6 linkage in

extracted glucan are Cc50, Cc119 and CSH1 and glucan extracted from all other isolates have 1—›3 linkage, 1—›6 linkage and β glycosidic bond.

Studies on β-glucan in *Fusarium* spp are relation to the caspofungin resistance. Glucan synthase genes reported in *Fusarium* are *fks-1* in *F. solani* (Ha *et al*., 2006), *gas1* in *F. oxysporum* (Caracuel *et al*., 2005). Mutation in *F. solani fks-1* gene leads to its resistance against antifungal agent caspofungin while *gas1* in *F. oxysporum* is required for virulence on plant system. To the best of our knowledge, studies related to the regulation of β-glucan synthesis are lacking. In current study, we report expression of glucanses and glucan synthase genes in FSSC. We were able to see highest expression of genes of G3 and FKS1 and GS2 in FSSC isolates. The genes G3 (glucanase) and GS2 (Glucan synthase) are yet to be characterized. It can be postulated that the levels of both, glucan synthases and glucanases govern the β-glucan synthesis in the cell wall. Several studies in other fungi e.g. *S. cerevisiae, Colletotrichum graminicola, C. albicans, Histoplasma capsulatum,* etc are discussed below to understand the importance of these enzymes in maintaining cell wall integrity.

β-glucan is important for cell survival. In one study, it was found that disruption of *KRE1* gene in *S. cerevisiae* caused reduction in cell wall β-1,6-glucan by 40%. Alteration was found in β-glucan structure in mutant strain. Further disruption of *KRE5* and *KRE6* genes also affected β-1,6-glucan production in cell wall and reduced β-1,6-glucan by 50%-80% (Boone *et al*., 1990). According to one another report, *KRE6* and *SKN1* gens are involved in β-1,6-glucan synthesis. Disruption of *SKN1* did not cause effect on β-1,6-glucan content but disruption of both the genes caused drastic decrease in cell wall β-1,6-glucan and cell morphology and structures were altered. *KRE6* and *SKN1* function independently but parallel in β-1,6-glucan synthesis (Roemer *et al*., 1993). Also it has been reported that in *Fusarium, gas1, fks1* and *fks2* mutation affects the β-1,3-glucan and β-1,6-glucan content in mutant strain. *gas1* mutation affected on β-1,3-glucan level in mutant but not β-1,6-glucan which indicate that *fks1* and *fks2* are important for both β-1,3-glucan and β-

1,6-glucan synthesis while *gas1* is involved in only β-1,3-glucan synthesis (Dijkgraaf *et al*., 2002).

In *Colletotrichum graminicola,* β-1,3-glucan synthase *GLS1* was down-regulated using RNA interference, as a result cell wall elasticity increased and appressoria was disrupted and cell wall was disrupted. It was concluded that β-glucan is required for cell wall rigidity in appressoria as well as for adhesion and differentiation while *GLS1* is required for appressorial penetration (Oliveira-Garcia and Deising, 2013).

In *C. albicans*, *BIG1* gene mutant showed reduced β-1,6-glucan content compared to wild type strain. To further check the effect of *CaBIG1* gene, the systemic candidiasis mouse model was developed for both the wild type and mutant strains and the adherence was checked on Human HeLa cell line. Mutant strain was not able to adhere to Human HeLa cells and colonization in kidney of infected mouse was reduced; the mutant rain was not able to from hyphae in infected mouse. This results suggests that *CaBIG1* gene is required for virulence of *C. albicans*. *CaKre5p* and *CaKre9p* mutant strains of *C. albicans* showed altered filamentation and both are involved in β-1,6-glucan synthesis in *C. albicans* (Umeyama *et al*., 2006). CaKRE6 and CaSKN1 mutation attenuate the virulence in mice. Also, deletion caused defect in cell wall, cell separation and hyphal formation in *C. albicans* (Han *et al*., 2019).

In *Histoplasma capsulatum*, two major β-glucanases, Eng1 and Exg8 are reported. Eng1 is responsible for removal of exposed β-glucan from surface of fungal cell wall as host Dectin-1 receptor binds to pathogen's exposed β-glucan and leads to phagocytosis. But Eng1 removes the glucan and hence imparts virulence to *H. capsulatum* while Exg8 has no role in virulence (Garfoot *et al*., 2017, 2016). Dectin-1 is not always important for defence against all pathogenic fungi. Dectin-1 knockout mice was infected with *C. albicans* and *Pneumocystis carinii*. The knockout mice infected with *P. carinii* was more susceptible to infection and formation of cyst in lungs was more. The knockout mice infected with low to higher dose of *C. albicans* and all the mice showed same survival with no significant difference in fungal burden in lungs. The immunocompromised Dectin-1 knockout mice also showed

survival. These results indicated that in *C. albicans* infection Dectin-1 is not important for protection against pathogen for host (Saijo *et al*., 2007). Later it was found that exposure of glucan remain masked from immune system because of mannan. Caspofungin treatment unmasked the glucan from yeast and hyphae and enhance binding of Dectin-1 to β-glucan (Graus *et al*., 2018; Lin *et al*., 2016).

Another important feature of pathogenic fungi is salvage mechanism or compensatory mechanism. It has been found that when *C. albicans* was exposed to caspofungin, a β-1,3-glucan synthase inhibitor, it starts formation of more chitin to compensate the glucan level and reduced the efficacy of drug (Walker *et al*., 2008). In A. fumigatus, *FKS1* repressed mutant strain when grown under repressed condition, the chitin content increased (Dichtl *et al*., 2015; Fortwendel *et al*., 2010).

Pleurotus ostreatus glucan synthase gene (*GLS*) expression was studied in wild type and overexpression strain. The GLS promoter was swapped with promoter of *GAPDH* gene of *A. nidulans* and expression of *GLS* increased compared to wild type. The β-glucan yield was also increased from 32% to 131% compared to wild type strain (Chai *et al*., 2013).

Expression of β-glucan synthase genes *PmBgs1* and *PmBgs2* from *Pholiota microspore* mycelium, premordium and fruiting body has been reported. Higher fold change of *PmBgs1* was detected in the dikaryotic mycelia and *PmBgs2* expression was higher in the primordia. This suggests that both the genes play distinct role at different stage of development (Zhu, 2018).

In *A. nidulans fksA* expression was studied during different stages of cell cycle. It was observed that *fksA* expressed more during G1, S and M phase. Also during asexual development level of *fksA* transcript was high but decreased after 22 hours and *StuA* is APSES transcription factor which negatively regulate *fksA* during asexual development which was proved by creating *StuA* mutant strain and in mutant strain the levels of *fksA* transcript and β-glucan contents significantly increased (Park *et al*., 2014). In *Coccidioides posadasii, FKS1* gene expression studied from early stage to

maturation stage. High expression of *FKS1* was seen in mycelial form and early spherulating stage but the expression decreased when spherules mature. Also, abnormal swelling of hyphae was observed upon deletion of *FKS1* (Kellner *et al*., 2005).

In *C. galbrata*, expression of *BGL2, XOG1, FKS1, FKS2, GAS2, KNH1, UGP1*, and *MNN2* genes was studied in biofilm in presence of antifungal drugs fluconazole, amphotericin B and caspofungin/micafungin. The gene expression of all genes up-regulated in presence of antifungal drug but overexpression was seen in presence of micafungin. Expression of genes involved in β-1,3-glucan biosynthesis, *BGL2, XOG1* and *GAS2* was highest among all other selected gens. It has reported that β-1,6-glucan and mannan are very important part of biofilm but in *C. glabrata* biofilm β-1,3-glucan is much important any play important role in biofilm resistance to antifungal (Rodrigues and Henriques, 2018).

In *S. cerevisiae,* three *FKS* genes, *FKS1*, *FKS2* and *FKS3* code for β-1,3-glucan synthase. Single deletion, double deletion and triple deletion strains were generated for all 3 *FKS* genes and the expression along with total β-glucan content and β-1,3-glucan content were studied. *FKS1* deletion up-regulated *FKS2* and *FKS3* gene expression. *FKS1* and *FKS2* deletion caused up-regulation of *KRE6* up to 4.9 fold which increased the β-1,6-glucan content to compensate loss of β-1,3-glucan in cell wall. Similar results for *KRE6* gene was obtained with triple deletion (*FKS1, FKS2* and *FKS3*) but other genes were down-regulated. Deletion of *FKS3* and *FKS1* caused up regulation of *FKS2, RHO1* and *RLM1*, which caused reorganization of cell wall. It can be concluded that *FKS1* and *FKS2* deletion are drastic for cell while *FKS3* deletion leads to increased stress resistance and viability of cell (Wang *et al*., 2018).

In order to understand the implications of the type and amounts of β-glucan in the mycelium, we studied the immunomodulatory effects using the whole blood assay. It is already known that different types of beta glucan elicit different responses with respect to cytokine production which depend upon structure, conformation, molecular weight and degree of branching. Noss and

group reported comparison of different types of glucan on whole blood assay to study their pro-inflammatory effect. They reported that lichenan (β-1,3-1,4-glucan), pustulan (β-1,6-glucan) and xyloglucan (β-1,4-glucan) induced highest TNF alpha production. Curdlan, barley beta glucan and yeast beta glucan induced strong to moderate production of TNF alpha and laminarin induced low TNF alpha production (Noss *et al*., 2013). It was reported that hyphal β-glucan and yeast β-glucan induce different immune responses (Erwig and Gow, 2016). *C. albicans* has unique close chain or cyclical structure which is not present in yeast glucan and hyphal glucan induced robust immune response compared to yeast glucan in human PMN cells (Lowman *et al*., 2014). Similarly, in current study we were able to see highest induction of TNF alpha production by curdlan. Also, FDSC glucan induced more TNF alpha production than FSSC glucan which indicates that there might be structural variation in both types of glucan and hence difference in their immunomodulatory effect.

To summarize, among the major 3 groups FSSC, FDSC and *F. sacchari* studied presently, the β-glucan concentration lies in narrow range in FSSC but in case of FDSC and *F. sacchari*, the β-glucan concentration varies highly among the studied isolates. To understand this disparity, study of β-glucan with more number of FDSC and *F. sacchari* isolates is required. Also, total β-glucan is higher in all isolates of *Fusarium* compared to β-1,3-glucan concentration and it might be concluded that much of β-1,3-glucan is being utilised in the formation of other forms of β-glucan in cell wall. Hence, NMR studies are required to prove other types of branches, if present. It has been reported that *fks* expression is dependent cell cycle of fungi (Park *et al*., 2014). There is a complex interplay of glucanase and glucan synthase genes in β-glucan biosynthesis. So we need to carry out more detailed day wise quantification and expression studies to get insight into role of genes in biosynthesis of β-glucan. Also, the factors which regulates the genes involved in regulating glucanase and glucan synthase genes need to be investigated in *Fusarium.*