Chapter 4

Physicochemical pretreatment of sugarcane bagasse for biomass deconstruction

4.1. Introduction:

Agrowastes from major cereal and pulse crops are being used as animal fodder. After Brazil, India is world's second largest producer and consumer of sugarcane (Bi et al., 2016). As mentioned in the Section 1.2 in Chapter 1, most of the sugarcane crop is consumed by sugar factories for production of sugar which yields a huge amount of sugarcane bagasse (SCB) as a by-product. SCB is the solid leftover fibrous material retained after crushing and squeezing the sugarcane to obtain its juice (Rocha et al., 2012). Biotechnological potentials of SCB agro residues has been described in detail by several investigators for their utilization in biorefinery for production of bioethanol, methane, and heat (Rabelo et al., 2011); in pulp and paper industries; production of alkaloids, several chemicals, metabolites and speciality enzymes during fermentation (Pandey et al., 2000); mushroom production, proteins enriched animal feed (Chandel et al., 2012; Mishra et al., 2014; Rahmani et al., 2014). Heat and steam, the products of cogeneration of bagasse, are traditionally consumed in sugar processing units to run boiler and turbine for electricity production (Mishra et al., 2014). Such biomass burning has recently raised several environmental and heath issues in India (Shepherd, 2017). In view of this, initiatives for process development of agricultural waste management without burning them have been taken by Government of India.

In Chapter 3, both xylano-pectinolytic enzymes obtained individually from three isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 significantly enhanced saccharification of barley husk, sugarcane bagasse and wheat husk when applied along with crude commercial cellulase. In industries also, depolymerization and saccharification of structural polysaccharides from plant biomass has been studied over past few decades. This process involved cellulase and other accessory enzyme mediated catalysis. After several efforts of commercializing it at plant level, the process is yet not completely optimized for 100% efficiency (Siqueira et al., 2017). Some of the common reasons for such observations were recalcitrance of plant cell wall, strong crystalline cellulose arrangements and unproductive binding of enzymes to several structural components like polysaccharides and/or lignin as well as enzyme inactivation over the incubation time and inhibition due to end-product accumulation (Kumar and Wyman, 2009b; Rocha et al., 2012). Recalcitrant nature of plant cell wall can be attributed to the complex interactions among cellulose, hemicellulose and aromatic lignin. Their use in biofuel production demands partial fractionation of cell walls and

maximum saccharification of cell wall polysaccharides (Mishra et al., 2014).

Thus, depolymerization of plant polysaccharides to fermentable sugar is the keystone step which limits the process of biofuel production from agro-waste biomass. This limitation can be overcome through physicochemical pretreatments of agro-waste biomass. Pretreatment increases biomass digestibility by improving the accessibility of glycosidic linkages to respective hydrolases and lyases. And the conversion of biomass to sugars through such physicochemical and enzymatic process is termed as biomass deconstruction (Blanch et al., 2011). Galbe and Zacchi, (2012) and many other researchers have suggested several processes and conditions for physicochemical pretreatments and how they affect the biomass to overcome the hurdles in saccharification. Pretreatments with mineral acids usually solubilize the hemicellulose bringing it in liquid fraction, leaving a pretreated fibrous solid rich in cellulose as well as lignin. The cellulosic polymers are rendered accessible to cellulase enzyme for improved saccharification due to hemicellulose removal and lignin relocation. Improved enzymatic saccharification of diverse biomass after pretreatment with acids have been reported for Sugarcane bagasse (Neureiter et al., 2002), Wheat straw (Agrawal et al., 2015), corn stover (Resch et al., 2014). Most of the cellulosic ethanol plants around the world use acid pretreatment technologies (Silveira et al., 2015). Alkaline pretreatments are efficient in removal of lignin while they don't solubilize any cellulose and hemicellulose fractions. Unaltered hemicellulose in solid biomass necessitates the requirement of accessory hemicellulases along with the core cellulases. Different agro wastes were treated in alkaline conditions and improved saccharification has been reported with rice straw (Zou and Shao, 2011) and SCB (C. A. Rezende et al., 2011). Ammonia fibre expansion and steam explosion treatment of corn stover (Yang et al., 2010), rice straw (Zhong et al., 2009), SCB (Rocha et al., 2012) has been investigated for lignin relocation or removal, the hydrolysis of hemicellulose which resulted in increased surface area for cellulose hydrolysis. Overall effects of other pretreatment techniques like organo-solvent, ionic solutions, hot water etc., used for pretreatments have been listed in Table 1.7 of the Chapter 1.

During the studies presented here, SCB was subjected to biomass deconstruction by physical agents like steam, heat and pressure; chemical reagents like ammonia, acid and alkali combined with steam and heat; followed by the enzymatic saccharification. Enzymatic saccharification of pretreated biomass revealed the effect and efficiency of pretreatments on biomass for their deconstruction.

4.2. Materials and Methods:

4.2.1. Chemicals, plant biomass, crude polysaccharide substrates, enzymes and their producer strains:

All required chemicals of analytical grade were purchased from HiMedia (Mumbai, India) or Sigma-Aldrich (Missouri, USA) or SRL Pvt. Ltd. (Mumbai, India). Sugarcane bagasse (SCB), an industrial waste biomass product, was selected for further pretreatment as well as enzyme cocktail mediated saccharification studies. Citrus peels (CP) and Wheat bran (WB) were collected, processed and stored as mentioned in Section 2.2.1 of Chapter 2 and were used individually as a crude polysaccharide substrate in media for selective production of crude xylanase and pectinase enzymes.

Three *Bacillus* cultures *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 obtained from camel, bull and buffalo dung respectively were maintained and stored on Nutrient Agar (NA) plates at 4-6 ºC. Xylanases and pectinases produced by these isolates as well as commercial cellulase Primafast®200 (Genencor, Du-Pont) were properly diluted and used as individual enzymes to study the biomass accessibility of pretreated biomass to these saccharifying enzymes.

4.2.2. Estimation of reducing sugar and calculation of % saccharification:

Released reducing sugar from biomass during different stages of studies such as washing, pretreatment and enzymatic saccharification was estimated using DNS reagent. 300 µl Di-nitro salicylic acid (DNS) reagent was added to 300 µl of the supernatant sample, collected during above mentioned stages and incubated in boiling water-bath for 10 min. Once the system was cooled down to room temperature, volume was made up to 1.5 ml by adding distilled water (DW) to each reaction system and absorbance was measured at 540nm on spectrophotometer (adapted and modified, Miller, 1959; Ghose and Bisaria, 1987). Released reducing sugar was quantified using D-glucose as standard.

% Saccharification can be calculated as follows by using the amount of reducing sugar released after hydrolysis per total provided substrate,

$$\%\, Saccharification = \frac{released\ reducing\ sugar\ (mg/ml)}{Substrate\ used\ (mg/ml)} \times 100 \qquad \text{(Eq. 4.1)}$$

4.2.3. Preparation of sugarcane bagasse (SCB) for pretreatment:

Squeezed long stem fragments of fresh sugarcane bagasse (SCB) were collected from the sugarcane processing unit located near Vadodara, Gujarat. Wet SCB was

comminuted in a grinder. 5 g of ground SCB was taken in 250 ml Erlenmeyer flask and washed with 100 ml warm (~60 °C) DW in shaking conditions at 120 rpm to remove soluble sugar present in the biomass. After every 1 h, the SCB biomass was filtered and fresh DW was added till the filtrate showed absence of reducing sugar as estimated in Sectuion 4.2.2. After this, ground, washed bagasse was collected back in several pre-weighed Petri dishes, and heat dried in a hot air oven at 60 °C till the weight remained constant for three subsequent measurements at one-hour interval. This dried and ground SCB was screened for its size and the fraction that could not pass theough the 0.5 mm sieve was separated and stored in airtight container at room temperature (Delabona et al., 2013). A part of this dried, ground and sieved biomass without any pretreatment was used as raw untreated control in further studies.

4.2.4. Pretreatment of SCB biomass:

Only SCB biomass was subjected to different physicochemical pretreatment methods i.e., i) Autoclaving ii) Steam Explosion iii) Alkali pretreatment, iv) AFEX pretreatment and v) Acid pretreatment were individually carried out in the autoclave vessel with loading capacity of 15.5 L under this study as mentioned below and the pretreated SCB henceforth will be referred as PSCB.

- ***Autoclave:***

Dried biomass of 1.25, 2.5 and 5.0% w/v were loaded in 250 ml Erlenmeyer flasks containing 100 ml of DW. After performing the pretreatment for holding time of 20 min at 10 PSI (115 °C) in an autoclave vessel the pressure was allowed to release slowly with time.

- ***Steam explosion:***

Dried biomass of 1.25, 2.5 and 5.0% w/v were loaded in 250 ml Erlenmeyer flasks containing 100 ml of DW. After holding the biomass for 5 min at 10 PSI (115 °C) in the autoclave, pressure valve was opened to suddenly release the pressure. Once the pressure reached to zero, the pressure valve was closed and the process was repeated thrice. Thus, pretreatment for total holding time of 20 min involving four steam explosions was completed.

- ***Alkali (NaOH):***

Three different concentrations of NaOH (1, 2 and 3% w/v) were individually used for this pretreatment. Individual set of three different biomass loadings containing

1.25, 2.5 and 5.0% w/v of dried biomass loaded in 250 ml Erlenmeyer flasks was separately subjected to each concentration of NaOH solution for pretreatment. After performing the pretreatment for holding time of 20 min at 10 PSI (115 °C) in the autoclave vessel the pressure was released gradually.

- ***AFEX (Ammonia Fibre Expansion, NH_4OH):***

Three different concentrations of NH_4OH (1, 2 and 3% v/v) were individually used for this pretreatment. Individual set of three different biomass loadings containing 1.25, 2.5 and 5.0% w/v of dried biomass loaded in 250 ml Erlenmeyer flasks was separately subjected to each concentration of NH_4OH solution for pretreatment. After performing the pretreatment for holding time of 20 min at 10 PSI (115 °C) in the autoclave vessel the pressure was released gradually.

- ***Acid (H_2SO_4):***

Three different concentrations of H_2SO_4 (1, 2, and 3 %v/v) were individually used for this pretreatment. Individual set of three different biomass loadings containing 1.25, 2.5 and 5.0% w/v of dried biomass loaded in 250 ml Erlenmeyer flasks was separately subjected to each concentration of H_2SO_4 solution for pretreatment. After performing the pretreatment for holding time of 20 min at 10 PSI (115 °C) in the autoclave vessel the pressure was released gradually.

Ratio of solid biomass to liquid (S/L) and ratio of solid biomass to pretreatment chemical reagent (S/CR) were calculated as below and further used to compare the effectiveness of pretreatment.

$$Solid\ biomass\ to\ lquid\ ratio\ = \frac{amount\ of\ solid\ biomass\ loaded}{amount\ of\ liquid\ used} \quad \text{(Eq. 4.2)}$$

$$Solid\ biomass\ to\ chemical\ reagent\ ratio = \frac{amount\ of\ solid\ biomass\ loaded}{amount\ of\ chemical\ agent\ used} \quad \text{(Eq.4.3)}$$

Biomass soaked in 100 ml DW at room temperature and pressure for 20 min without any physicochemical pretreatment was used as control throughout the studies presented in this chapter. After completion of pretreatment, all samples were allowed to cool at room temperature and filtered through the nylon sieve filter. The remaining filtrate was separated from biomass through centrifugation at 10,000 rpm for 20 min. This collected filtrate was stored at -20 °C till next analysis, while solid biomass was directly subjected to further analysis.

4.2.5. Effect of pretreatment:

Analysis of i) Filtrate and ii) Pretreated sugarcane bagasse (PSCB) biomass was performed to evaluate the effect of pretreatment. Filtrate was analysed for release of soluble reducing sugars (SRS) while pretreated biomass was assessed for structural as well as chemical changes and its enzymatic digestibility.

4.2.5.1. Analysis of filtrate for released soluble reducing sugars:

Total released soluble reducing sugar (SRS) from filtrate was quantified with DNS method while the chromatographic techniques were further used to know the type of released SRS.

4.2.5.1a. Estimation of reducing sugar:

The amount of released SRS during pretreatment of SCB was estimated by DNS method given in Section 4.2.2. SRS was expressed in terms of % released SRS to compare the effect of different pretreatments as mentioned below.

$$\%\ released\ SRS = \frac{sugar\ released\ during\ pretreatment\ (mg)}{biomass\ used\ for\ pretreatment\ (mg)} \times 100 \qquad \text{(Eq. 4.4)}$$

4.2.5.1b. Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) analysis:

20 µl of aliquots were withdrawn from each type of pretreatment filtrate and was loaded on Silica Gel 60G and fluorescent indicator F254 containing aluminium plates for TLC as well as in Hi-plex-H column for HPLC. 5mM D-Xylose and 5mM D-glucose were used as standards and samples were analyzed as follows.

- ***TLC analysis***

Filtrate samples were individually loaded on TLC plates coated with Silica Gel 60G and fluorescent indicator F254. Water-butanol-methanol in 1:4:5 ratio was used as mobile phase. Carbohydrates spots were detected on the chromatogram by spraying with alcoholic p-Anisidine phthalate solution (1.23 g p-Anisidine and 1.66g phthalic acid in 100ml 95% ethanol) and thereafter gently heating the plate for 15-20 mins at 60°C (Kurt Randerath, 1963).

- ***HPLC analysis:***

From withdrawn aliquote, 20 µl of samples was loaded in Hi-Plex-H column specific for carbohydrates and alcohols on Shimadzu HPLC system equipped with LC-10AT pump, CTO-10ASVP oven column cabinet, and detected using RID-10A detector. Individually, D-glucose, D-xylose and D-galacturonic acid monomers, were

also loaded as control. The stationary phase in Agilent Hi-Plex-H column has strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene co-polymer in hydrogen form with diameter of 7 to 11 µm and deionized water was used as mobile phase for separation of hydrolysate products under isocratic conditions as recommended by Agilent. Chromatographs for individual sample were compared with the monomer controls with respect to retention time and peak area.

4.2.5.2. Analysis of pretreated biomass:

Pretreated biomass was analyzed by **(a)** Gravimetric analysis for loss of dry weight, **(b)** Scanning electron microscopy (SEM) for alteration in structural composition, and **(c)** Fourier-Transform Infrared Spectroscopy (FTIR) for chemical analysis.

4.2.5.2a. Gravimetric analysis:

Solid pretreated biomass obtained after filtration was washed several times with fresh DW to neutralize the pH, squeezed and collected in pre-weighed Petri dishes and morphology of untreated and treated biomass was recorded visually. After that biomass was heat dried in a hot air oven at 60 °C till the weight remained constant for three subsequent measurements at one-hour interval. The dried biomass was stored in air tight container at room temperature and used for further analysis. Decrease in weight of dry biomass was calculated as mentioned below and expressed as % weight loss in biomass.

$$\%\, weight\ loss\ in\ biomass = \frac{biomass\ obtained\ after\ drying\ (mg)}{biomass\ used\ \ for\ pretreatment\ (mg)} \times 100 \qquad \text{(Eq. 4.5)}$$

Further, the amount of soluble components other than released SRS, removed due to pretreatment from biomass was also calculated using the following formula.

$$\%\ Other\ soluble\ components = (\%\ Weight\ loss\ in\ biomass - \%\ Released\ SRS) \qquad \text{(Eq. 4.6)}$$

4.2.5.2b. Fourier-Transformed Infrared Spectroscopy (FTIR):

Fourier Transform Infrared (FTIR) spectroscopy was carried out for both untreated and PSCB biomass to reveal the functional groups and their band intensity, stretching vibrations and absorption peaks that contribute to the cellulose, hemicellulose and lignin structure. All solid samples were air dried and sent to the FTIR lab at Central Research Facility (CRF), IIT-Kharagpur, West Bengal, India. Samples were mixed with potassium bromide (KBr) and then pressed into a disc form. Spectra of FTIR were obtained over the range of 400-4000 cm^{-1} with a spectral resolution of

0.5 cm^{-1} (Magalhães da Silva et al., 2013; Rajak and Banerjee, 2015).

4.2.5.2c. Scanning Electron Microscopy (SEM):

The selected biomass samples were collected in a microfuge tube and washed with Phosphate buffered saline (PBS) pH 7.2 ± 0.2, fixed in 2.5% v/v glutaraldehyde for 15 min, again washed with PBS and dehydrated in a series of increasing acetone concentrations i.e., 10, 25, 50, 75 and 100% for 10 min each and stored in 100% absolute acetone at -20 °C till further analysis. (adapted and modified, Chutani and Sharma, 2016). For imaging process, sample of the biomass was air dried and placed on an adhesive carbon tape fixed on metal stub and sputter coated with Platinum (Pt) in Auto Fine Coater (JEOL-JFC-1600). The structure of plant cells in the biomass was examined at 10kV under SEM (JEOL, JSM-7600F-FEG-SEM) at Sophisticated Analytical Instrument Facility (SAIF), IIT-Powai, Mumbai, India. Electron micrographs were taken at desired magnifications.

4.2.6. Enzyme preparation for saccharification studies:

B. safensis M35, *B. altitudinis* R31 and *B. altitudinis* J208 were inoculated in BHM-YEP media containing either CP or WB for production of pectinase or xylanase respectively as mentioned in Section 3.2.2 of Chapter 3. Thus, obtained cell free supernatant was used as source of crude pectinase or xylanase and henceforth will be referred to as M35 xylanase, M35 pectinase, R31 xylanase, R31 pectinase, J208 xylanase and J208 pectinase throughout the studies. Primafast®200, a commercial source of cellulase will be referred to as commercial cellulase.

4.2.7. Accessibility of polysaccharides from pretreated biomass for their enzymatic saccharification:

To study how the pretreatments have changed polysaccharide accessibility of SCB, enzymatic digestibility for each PSCB was analyzed by subjecting it to different enzymes, i.e., commercial cellulase, M35, R31 and J208 xylanases as well as M35, R31 and J208 pectinases individually and hydrolysate was further analysed.

4.2.7.1. Estimation of reducing sugar in enzymatic hydrolysate of PSCB biomass:

PSCB at biomass loading of 2% w/v in 10 ml of 50 mM Tris-Cl pH 7.0 buffer was digested separately by commercial cellulase, M35, R31 and J208 xylanases as well as M35, R31 and J208 pectinases individually. 320 µg of commercial cellulase (C) and 400 µg for individual xylanase (X) or pectinase (P) were loaded per 200 mg of dry PSCB substrate. The system was amended with 100 µg/ml of Sodium azide ($Na\text{-}N_3$),

Ampicillin, Kanamycin and Streptomycin each to prevent microbial contaminations and was incubated on shaker at 160 rpm, 40 °C for 60 h. Released reducing sugars were estimated after 60 h with DNS method as mentioned in Section 4.2.2. Accessibility of the polysaccharide substrate was calculated in terms of % saccharification achieved by each enzyme individually.

$$\%\ Saccharification\ = \frac{reducing\ sugar\ released\ by\ enzymatic\ hydrolysis\ (mg)}{Initial\ solid\ biomass\ used\ for\ hydrolysis\ (mg)} \times\ 100 \quad \text{(Eq.4.7)}$$

4.2.7.2. Fluorescence microscopic analysis of pretreated biomass anatomy during cellulase activity:

A separate experiment was performed where 0.5x0.5x1.0 cm^3 sized cuboid pieces were cut from the pith of fresh sugarcane stem. Individual pieces were washed thoroughly with water till removal of soluble sugars ceased. Two of these pieces cumulatively weighing 1.25 g together were exposed to the different pretreatments as mentioned in section 4.2.4. The pretreated cuboidal pieces were washed thoroughly with water to neutralize their pH followed by different pretreatments. Thin hand-cut transverse sections from the untreated and pretreated cuboid stems were selected and light and fluorescence based anatomical studies to understand the pretreatment effect on plant cell wall digestibility by commercial cellulase.

The selected thin sections of untreated and pretreated samples were stained with 0.1% Safranin-O (prepared in 1% Ethanol), washed with water at 30 °C and mounted on clean glass slides with the 100 µl of properly diluted (50 µg/100µl) commercial cellulase (Bond et al., 2008; Sant'Anna et al., 2013). Light and fluorescence microscopic observation of this cellulase treated sections on Olympus CX-41 system microscope was performed with help of CX-RFL-2 reflected fluorescence attachment. Two of the reflected light fluorescence mirror cubes CX-DMB-2 and CX-DMG-2 were used. CX-DMB-2 possessed the excitation filter BP475 (475nm, blue colour range for excitation) and a dichroic mirror DM500 with barrier filter O515IF which allows observation in range of (500-515nm) green fluorescence colour. CX-DMG-2 possessed the excitation filter BP545 (545nm, green colour range for excitation) and a dichroic mirror DM570 and barrier filter O590 which allows observation in range of (570-590nm) orange-red fluorescence colour. , The later one, i.e., CX-DMG-2 was modified for the excitation filter. Keeping the dichroic mirror DM570 and barrier filter O590, the excitation filter BP545 was replaced with BP475 to obtain the excitation in range of blue colour wavelength and to observe the emission in range of orange-red colour

wavelength. The images were taken after every 15 min and compared for gradual disappearance of cellulose from various region of the sugarcane stem transverse section (TS).

4.2.8. Data analysis:

- All the experiments were performed thrice and for quantification experiments Data-values and Errors in the result tables or in graphs are represented as Mean and Standard Error of Mean (Mean ± SEM) in either GraphPad Prism 6.0 (San Diego, CA, USA) or Origin 2017 (Northampton, MA, USA).
- Statistical analysis was carried out using the Two-Way ANOVA method in Geaphpad Prizm.
- FTIR Data was analyzed using EFTIR software and graphs were plotted using Y-offset graphs in Origin 2017.

4.3. Results and Discussion:

According to the review by Galbe and Zacchi (2012) different combinations of physical conditions like temperature, pressure and time along with water or chemical reagents increases the accessibility and digestibility of cellulose as well as hemicellulose by reducing matrix complexities and cellulose crystallinity. An efficient pretreatment should result in high recovery of all carbohydrates, high digestibility of the cellulose during the subsequent enzymatic hydrolysis, high solids concentration as well as least concentration of liberated sugars in the liquid fraction, production of no or very limited amounts of sugar and lignin degradation products. Besides this, the process should also avail low energy and operational cost, and capital investments.

With focus on improvement of SCB utilization as raw material in biofuel production industries, the effect of physicochemical pretreatment methods on gravimetry, chemical and structural composition as well as enzymatic digestibility of SCB biomass were studied and analyzed as ahead.

4.3.1. Factors governing pretreatment process:

SCB biomass is used as a sustainable energy source because of its lignocellulosic nature (Pandey et al., 2000). Reduction of particle size for agrowaste biomass is often performed to make material handling easier and to increase surface area to volume ratio resulting in better accessibility of substrates and enhances enzymatic saccharification (Silveira et al., 2015). This can be achieved through

mechanical pretreatment by one of the methods like chipping, comminution or milling and followed by washing with DW for removal of soluble materials to decrease their hindrance in the subsequent saccharification studies. As discussed in Section 4.1 disruption of specific components from lignocellulosic matrix through diverse physicochemical or mechanochemical pretreatments alters the complexity of the matrix, making it loose and more porous (Galbe and Zacchi, 2012; Silveira et al., 2015; Terán Hilares et al., 2018). This helps the cell wall degrading enzymes to enhance their saccharification activity and determines that how each pretreatment method is efficient in terms of improving biomass saccharification activity.

Autoclave, steam explosion, alkali, AFEX and acid pretreatments were performed with SCB biomass in order to improve its enzymatic digestibility. Physical factors like heat (temperature), pressure (P) and time (t) as well as other factors like solid biomass to liquid ratio (S/L, biomass loading) and solid biomass to chemical ratio (S/CR, chemical loading) etc., affect the pretreatments. Temperature, time and pressure were maintained constant for the studies to investigate their effects on the biomass digestibility (N. S. Mosier et al., 2005; Alvira et al., 2010). For all five pretreatment methods, the ratio of solid biomass loading to liquid (S/L) and ratio of solid biomass loading to chemical reagents (S/CR) were calculated from the equations mentioned in Section 4.2.4 and values are presented below in Table 4.1. S/L ratio of 1:80, 1:40 and 1:20 for solid biomass loading of 1.25, 2.5 and 5.0% SCB was maintained constant and used in all physicochemical pretreatment methods. Treatment of 1.25, 2.5 and 5.0% SCB biomass loadings with 1, 2 and 3% of each chemical reagent (NaOH, NH_4OH, H_2SO_4 individually) creates a matrix with S/CR ratio values of 1:0.2 to 1:2.4.

Table 4.1. S/L and S/CR loading ratios for different physicochemical pretreatments:

Solid biomass (SCB) loading (% w/v)		1.25%	2.5%	5.0%
S/L Ratio at liquid loading of 100 ml DW		**1:80**	**1:40**	**1:20**
S/CR Ratio at chemical reagent loading of NaOH (% w/v), NH_4OH (% v/v), H_2SO_4 (% v/v)	1%	**1:0.8**	**1:0.4**	**1:0.2**
	2%	**1:1.6**	**1:0.8**	**1:0.4**
	3%	**1:2.4**	**1:1.2**	**1:0.6**

Combinations of biomass and one or more chemical reagents in diverse S/L ratio or S/CR ratio were used for pretreatment along with modifications in residence or holding time and temperature-pressure conditions have been widely studied and summarized by Hendriks and Zeeman (2009). Li et al., (2014) has used 1:1 liquid to

solid ratio for SE pretreatment of SCB. Alizadeh et al., (2005) pretreated switchgrass with AFEX treatment having ratio of 1:1 chemical reagent (ammonia) to solid biomass at ambient to 120 °C temperatures ranging from few days to several min. da Silva Martins et al., (2015) pretreated different loadings of SCB with dilute acid ratio while Maryana et al., (2014) has reported the effects of pretreatments on SCB with different ratio of SCB biomass to NaOH loadings.

4.3.2. Morphological changes in SCB:

Visual differences between the morphology of raw and PSCB biomass by individual pretreatment methods are represented in the Figure 4.1. Both autoclave (10 PSI or 68.95kPa, 117 °C for 20 min) and steam explosion (10 PSI or 68.95 kPa, 117 °C for 5 min x 4 times) treatments enhanced the opacity as well as softness of biomass. Steam explosion imparted brighter, whitish and fluffy appearance to SCB.

Garmakhany et al., (2014) have demonstrated the conversion the Canola straw to fine powder during intense steam explosion conditions (900 kPa, at 180 °C for 4 min) in a commercial steam explosion plant. In another studies with duckweed (*Lemna minor*) Zhao et al., (2015) also showed the enhancement in tissue disruption at higher pressure (0.2-2.7 MPa) and temperature (130-230 °C) with holding time of 10 min. So, the conditions we have used for hydrothermal pretreatments like steam explosion and autoclave in present studies might not have completely broken down the bagasse biomass as it is not reduced to powder, yet they have brought certain changes in morphology.

Chemical pretreatment methods altered bagasse biomass morphology drastically by loosening the soft, fluffy pith region from the fibrous transport elements and gave more fibrous appearance to the biomass (Figure 4.1 D-F). Acid pretreatment imparted the pink-red colour due to formation of sugar furfurals during reaction with by-products of lignin. NH_4OH and NaOH treatments have imparted yellow colouration in an increasing order in turn with increased concentration of alkali. Similar changes in morphological appearance of SCB was observed after NaOH and $CaCl_2$ pretreatment (Utomo et al., 2015). Similar to the observations for bagasse biomass, increase in opacity for steam explosion and autoclave methods as well as loosening of the soft pith region from the fibrous transport elements and fibrous appearance in NaOH, AFEX and H_2SO_4 methods were also found from cuboid sugarcane stem after the pretreatments.

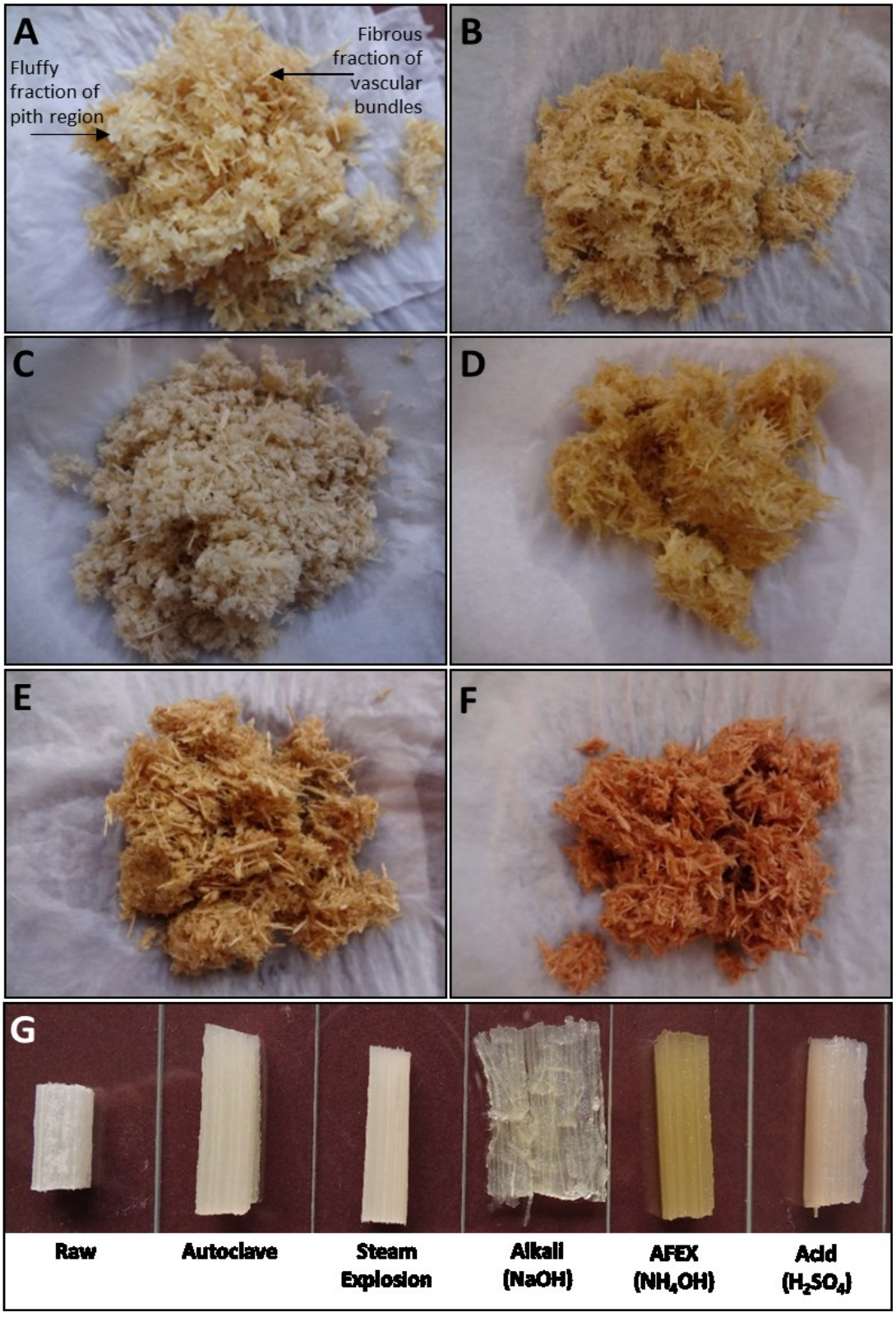


Figure 4.1. Visible morphological differences in raw and PSCB biomass:
(A-F): Morphological appearance of ground and fractionated SCB biomass **(A)** Raw-untreated SCB; and SCB pretreated with **(B)** Steam explosion, **(C)** Autoclave, **(D)** Alkali (NaOH), **(E)** AFEX (NH_4OH), and **(F)** Acid (H_2SO_4) methods at 5.0% SCB loading and 3.0% chemical loading as mentioned in Section 4.2.4; **(G)**: Morphological appearance of intact pith cuboids from SCB stem before and after pretreatments of 1.25% SCB loading and 1.0% chemical loading as mentioned in Section 4.2.7.2.

4.3.3. Biochemical analysis of filtrate for SRS:

Effect of pretreatment on biomass was evaluated by analyzing the release of SRS. Amount of SRS released from raw and pretreatment filtrate is depicted in Figure 4.2. Less than 0.1% SRS were detected from filtrate of untreated biomass. Two-Way ANOVA at confidence interval of 99.9% ($p < 0.001$) suggested that filtrate from all pretreatment methods exhibited significantly higher counts of the SRS than untreated filtrate (significance levels not presented in figures). The release of SRS increased in the following order of pretreatments: NaOH, Autoclave, Steam explosion, NH_4OH and H_2SO_4. During Autoclave, SE and NaOH treatments, 0.2-0.5% SRS was released, which increased to the range of 0.4-0.8% for NH_4OH treatment and further to the range of 4.0 -8.0% for H_2SO_4 treatment. For all treatments, when released SRS were compared at different SCB loadings, 1.25% SCB loading exhibited maximum amount of released SRS. Two-Way ANOVA analysis suggested that, the difference in released SRS for 1.25% and 2.5% SCB loadings was significant for all pretreatments but that between 2.5% and 5.0% was only significant for H_2SO_4 pretreatments.

TLC and HPLC analyses for cellulosic and hemicellulosic components (i.e., glucose and xylose) released from biomass is presented respectively in Figure 4.3 and Figure 4.4. Neither xylose nor glucose were detected in autoclave and steam explosion, NaOH and NH_4OH pretreatment filtrates as their amount was below detection level (Figure 4.3a). For H_2SO_4 pretreatment intense spots corresponding to xylose standard (red box) were developed as compared to glucose (purple box) (Figure 4.3b). As seen in Figure 4.4, HPLC analysis clearly discriminated between the released SRS. Glucose and xylose were completely absent in autoclave (Figure 4.4B) and steam explosion (Figure 4.4C) filtrate. Glucose was detected (RT 11.4 min) in NaOH (Figure 4.4D) and NH_4OH (Figure 4.4E) filtrate, while higher amount of xylose (RT 12.1 min) was detected than glucose in H_2SO_4 pretreatment filtrate, (Figure 4.4F).

Thus TLC and HPLC analyses suggested that there is an appreciable release of hemicellulose in filtrate during H_2SO_4 pretreatment. Carvalho et al., (2015) had reported unaltered cellulose content from hydrothermally, alkali and acid pretreated biomass whereas decreased hemicellulose content in acid pretreated biomass instead of filtrate. Similarly, Maryana et al., (2014) have reported increased fraction of cellulose content with minor decrease in hemicellulose content from recovered dry SCB after NaOH pretreatment. These observations indirectly suggested that NaOH didn't affect major sugar components but H_2SO_4 dissolved major hemicellulose content.

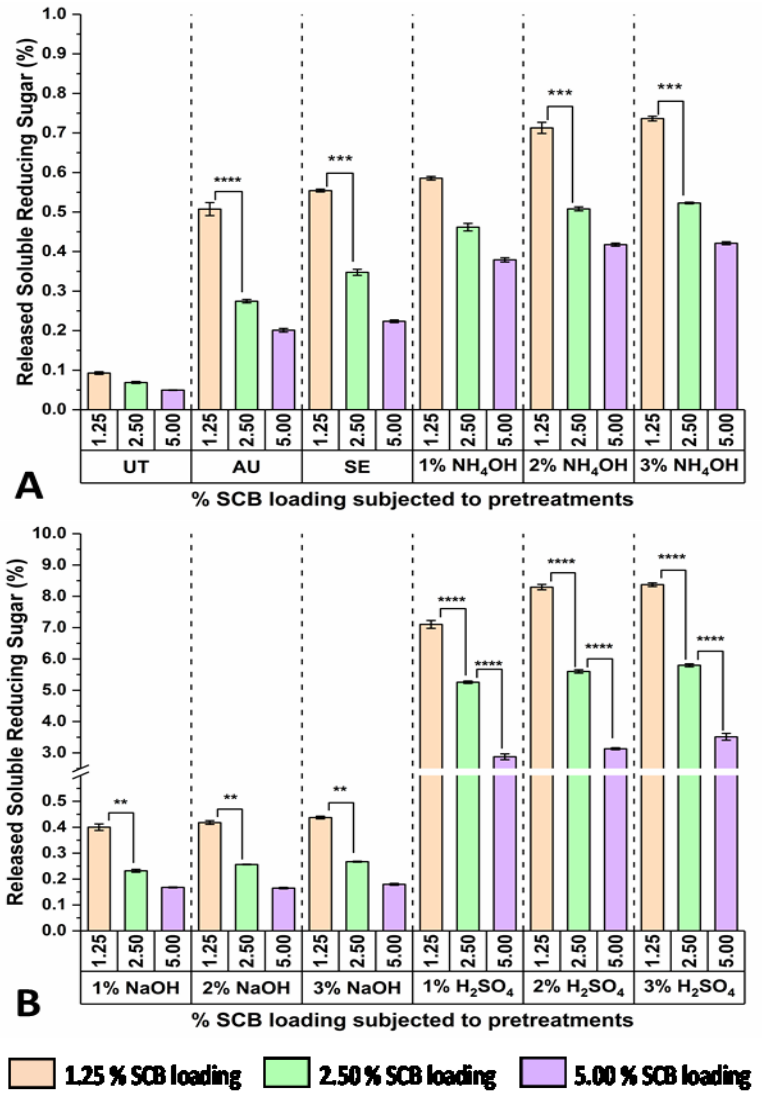


Figure 4.2. Release of % SRS in pretreatment filtrate from raw and PSCB:
(A) Raw untreated control, Autoclave, Steam explosion, and NH_4OH pretreatments; **(B)** NaOH and H_2SO_4 pretreatments; Significance difference given as * = $p < 0.05$, ** $p < 0.01$, *** =$p < 0.001$ and ****= $p < 0.0001$; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

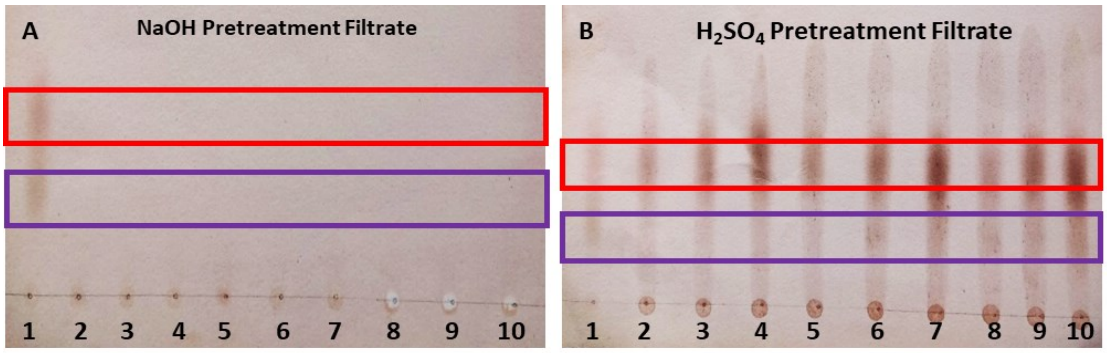


Figure 4.3. Representative chromatographs of TLC analysis for pretreatment filtrates:
(A) NaOH filtrate analysis; **(B)** H_2SO_4 filtrate analysis; **Loaded spots 1-10 are as, (1):** Standards (**xylose in red box** and **glucose in purple box**); **(2, 3 and 4):** 1.25, 2.5, and 5.0% SCB treated with 1% chemical reagent; **(5, 6 and 7):**1.25, 2.5, and 5.0% SCB treated with 2% chemical reagent; **(8, 9 and 10):** 1.25%, 2.5%, 5.0% SCB treated with 3% chemical reagent.

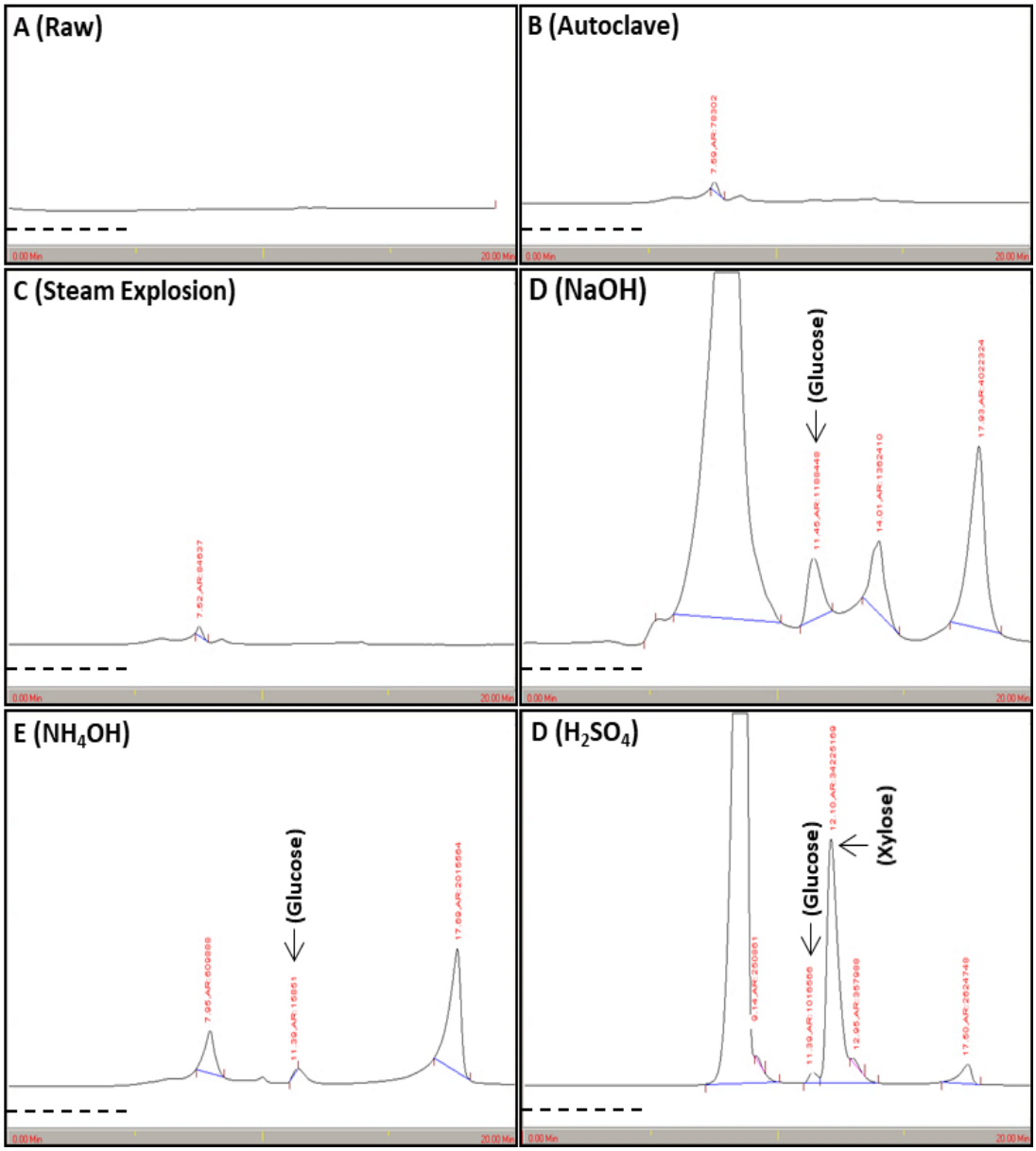


Figure 4.4. Representative chromatographs of HPLC analysis for pretreatment filtrates: (A-F): HPLC Chromatograph of 5% (w/v) loading of SCB **for (A)** Raw untreated control; **(B)** Autoclave, **(C)** Steam explosion, **(D)** 3% NaOH, **(E)** 3% NH_4OH and **(F)** 3% H_2SO_4; RT: Glucose-11.4 min, Xylose 12.1 min; Dashed line (---) at X-axis indicates the time span of 5 min on chromatogram; Images presented are representative ones from n=3.

4.3.4. Gravimetric analysis of pretreated biomass:

Figure 4.5. depicts % loss in dry weight of SCB biomass after pretreatment. Minimum of 0.7-0.9 % loss in dry weight was observed in case of raw biomass used as untreated control. Two-Way ANOVA at confidence interval of 99.9% ($p < 0.001$) suggested that all pretreatments significantly enhanced % loss in dry weight (Significance levels not presented in figures). 25 to 45 % biomass loss was observed for NaOH as well as H_2SO_4 pretreatment and NH_4OH exhibited 10 to 16% weight loss. Autoclave and steam explosion exhibited 10-13% and 5-6% weight loss respectively.

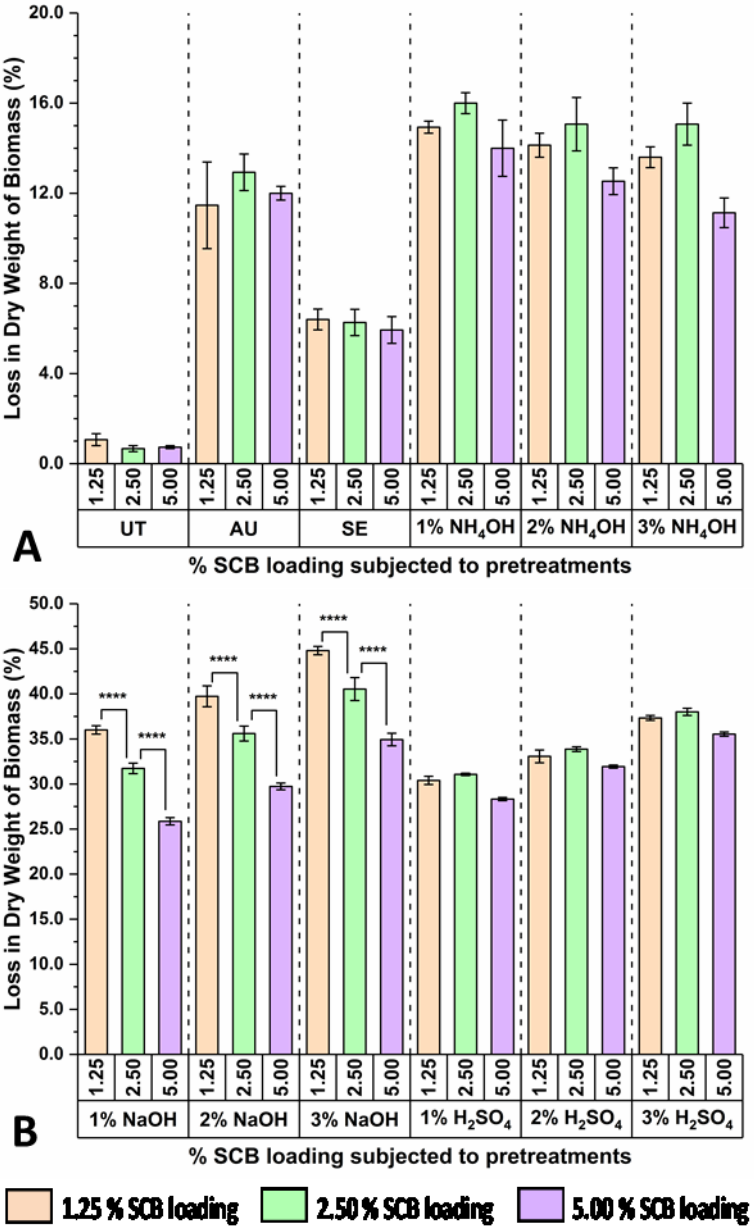


Figure 4.5. Loss in dry weight (%) from raw and pretreated biomass:
% Loss in dry weight from **(A)** Raw untreated control, Autoclave, Steam explosion and NH_4OH pretreatments; **(B)** NaOH and H_2SO_4 pretreatments; Significance difference given as * = $p < 0.05$, ** $p < 0.01$, *** =$p < 0.001$ and ****= $p < 0.0001$; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

Highest % weight loss was observed at lowest SCB loading i.e., 1.25% SCB in case of untreated control as well as for SE and NaOH pretreatments and as the SCB loading increased, the % weight loss decreased. Whereas, for Autoclave, NH_4OH and H_2SO_4 pretreatments, highest % weight loss was observed at SCB loading of 2.5%. Two-Way ANOVA of % biomass loss suggested that there is a significant difference between % biomass loss for each SCB loading in NaOH pretreatment ($p < 0.001$). These observations corroborate the earlier reports where Carvalho et al., (2015) has reported

15.4, 49.3 and 49.1 % loss in dry weight of SCB biomass due to hydrothermal, acidic and alkaline pretreatments respectively and also reported a similar pattern of weight loss for eucalyptus and sugarcane straw biomass.

- ***Effect of biomass and chemical loadings in chemical pretreatment on % release of SRS and loss in dry weight:***

As could be seen from Table 4.1, three different biomass loadings along with three chemical loadings created a matrix of nine different combinations individually for NaOH, NH_4OH and H_2SO_4 treatments. Hence, the effect of biomass and chemical loadings on SRS and % loss in dry weight was studied to understand how the change in biomass loading and chemical loading affected the weight loss and released soluble reducing sugars. Based on these combinations, the contour plots for % released SRS (Figure 4.6 A-C) and % dry weight loss (Figure 4.6 D-F) for individual treatments were plotted and analyzed.

NaOH, NH_4OH and H_2SO_4 is the pretreatment order in which overall release of SRS was increased (Figure 4.6 A-C). Release of SRS during all three chemical pretreatments followed similar pattern, where it increased with enhanced chemical loadings of NaOH, NH_4OH and H_2SO_4 individually and decreased with enhanced biomass loadings (Figure 4.6 A-C).

NH_4OH, H_2SO_4 and NaOH is the pretreatment order in which overall % dry weight loss of SCB was increased (Figure 4.6 D-F). Unlike the release of SRS, the pattern of % loss in dry weight was drastically different during each chemical pretreatment. During alkali pretreatment dry weight loss increased with enhanced NaOH loadings and decreased with enhanced biomass loadings (Figure 4.5 D). During AFEX pretreatment dry weight loss decreased with enhanced NH_4OH loadings. Dry weight loss increased initially when biomass loading was 2.5%, then decreased at 5.0% biomass loading (Figure 4.5 E). During acid pretreatment, dry weight loss increased with enhanced H_2SO_4 loadings. Weight loss also increases initially when biomass loading was 2.5%, then it decreased at 5% biomass loading. (Figure 4.5 F).

Thus, the different patterns of % released SRS and % loss in dry weight, along with detection of several peaks in HPLC chromatograms (other than glucose and xylose peaks observed at ~11.4 and ~12.1 min respectively, Figure 4.4). suggested there must be other components which were solubelized in alkali, acid or water and released from biomass in filtrate during these pretreatments.

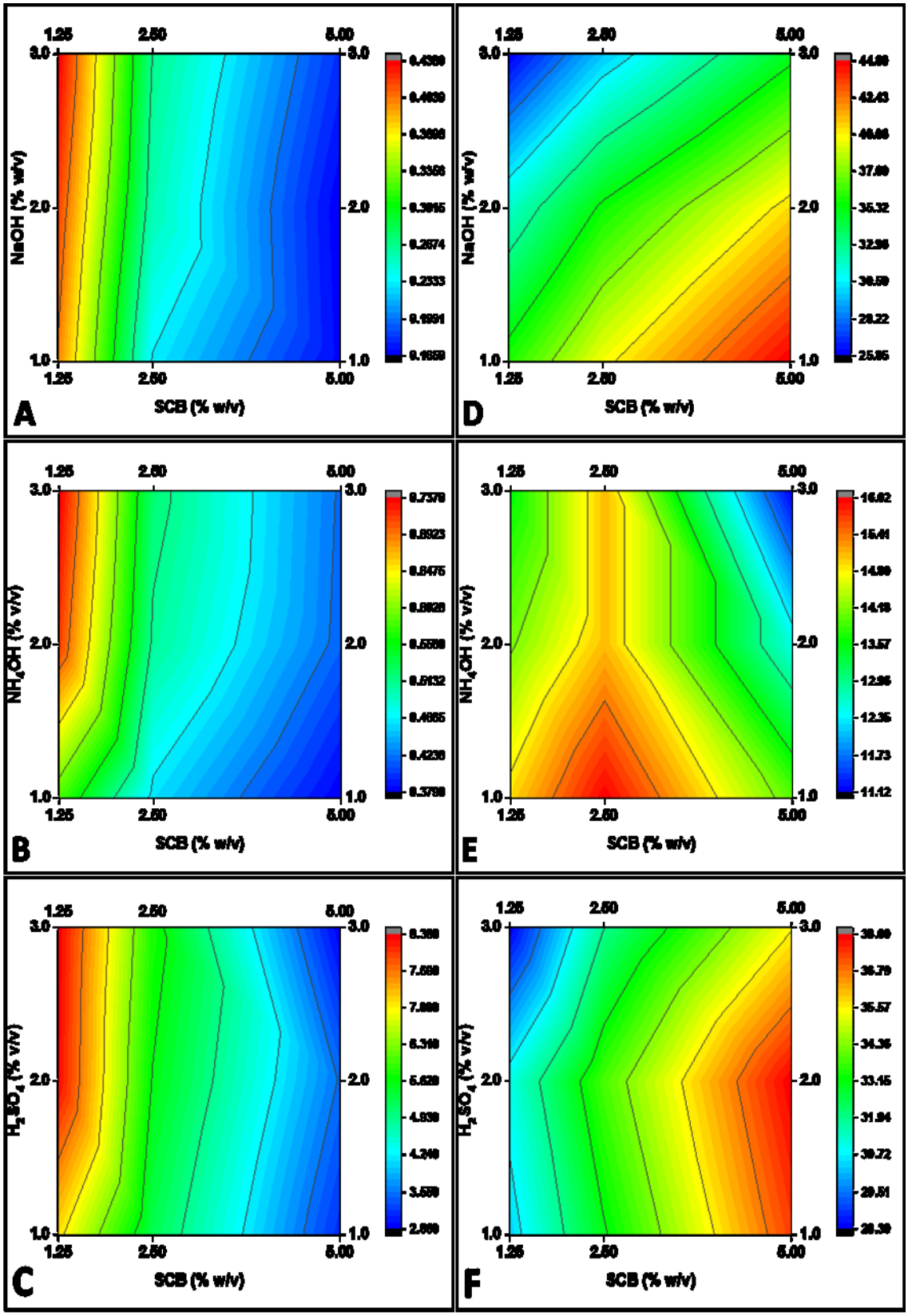


Figure 4.6. Contour plots representing release of reducing sugar and dry weight loss at different loading values of biomass and chemicals:
Upper panel (A-C): % released soluble reducing sugar due to **(A)** NaOH, **(B)** NH_4OH and **(C)** H_2SO_4 pretreatment; **Lower panel (D-F):** % loss in dry weight of biomass due to **(D)** NaOH, **(E)** NH_4OH and **(F)** H_2SO_4 pretreatment.

4.3.5. Analysis of soluble components other than SRS released from SCB:

The difference between amount of released SRS and % dry weight loss was expressed as the amount of solubles other than released reducing sugars in Table 4.2. Release of these soluble components was significantly higher in case of each pretreated biomass from individual treatments than raw untreated control biomass (ANOVA at $p < 0.001$). All pretreatments except NaOH exhibited more release of other solubles at 2.5% loading of biomass than 1.25% and 5.0% loadings. Whereas NaOH exhibited correlation ($R^2 = 0.99$) as it released highest solubles at 1.25% SCB loading, which further decreased with increased biomass loading. While released soluble components increased with increase of NaOH and H_2SO_4 loadings, it decreased with increase of NH_4OH loadings.

Presence of different peaks in HPLC chromatogram from NaOH, NH_4OH and H_2SO_4 pretreatment filtrate also suggested release of several solubles components from SCB biomass along with glucose and xylose). More number of peaks were observed for NaOH, H_2SO_4 and NH_4OH indicating the release of diverse solubles. Li et al., (2016) suggested that the major proportion of this solubles in pretreatment filtrate of NaOH and H_2SO_4 is occupied by lignins.

Table 4.2. % Solubles released from biomass other than SRS during different pretreatments:

SCB (% w/v)	Pretreatment											
	Control	AU	SE	NaOH (% w/v)			NH_4OH (% v/v)			H_2SO_4 (%v/v)		
				1	2	3	1	2	3	1	2	3
1.25	0.71	10.96	5.71	**35.60**	**39.32**	44.36	14.35	13.42	12.86	23.30	24.77	28.96
2.50	0.73	**12.66**	**5.92**	31.50	35.34	40.27	**15.54**	**14.56**	**14.54**	**25.81**	**28.80**	**32.20**
5.00	**0.75**	11.80	5.81	25.70	29.57	34.75	13.62	12.12	10.71	25.46	28.27	32.02

AU-Autoclave; SE-Steam explosion; Values in bold indicates the highest values; Values presented are Mean for n=3.

These results suggested that though NaOH and H_2SO_4 exhibited higher amount of biomass loss followed by NH_4OH pretreatment, the fraction of soluble reducing sugar was least in case of NaOH pretreatment and highest for H_2SO_4 pretreatment, whereas the amount of other solubles was highest in case of NaOH followed by H_2SO_4 and NH_4OH treatments. Although during steam explosion and autoclave pretreatments, the solubles like lignin were not removed in large amount, the treatments certainly brought morphological changes. Kristensen et al., (2008) and Siqueira et al., (2017) had suggested that, though hydrothermal pretreatment couldn't remove lignin they

structurally relocate it on the surface of the cell wall creating several micropores in the matrix which increased the accessibility of cellulose and hemicellulose to their respective saccharifying enzymes. As explained by Galbe and Zacchi, (2012), the pretreatment which releases less amount of soluble reducing sugars and more amount of solubles other than reducing sugars is more preferable for preapartion of raw material in bioethanol industries. Because, the carbohydrate polysaccharide content is still intact and lignin polymers are removed. These conditions favour the enhancement in saccharification of biomass.

The comparison of released reducing sugars, with soluble components other than SRS and weight loss suggested that, NaOH, NH_4OH, Steam explosion, Autoclave and Steam Explosion is the order in which the pretreatments should favour the saccharification. In literature also, several reports have been cited suggesting genetic modification of microorganisms for utilization of pentoses and hexoses for enhanced production of bio-alcohols (Atsumi et al., 2008; Bokinsky et al., 2011; Nguyen et al., 2016; Avanthi et al., 2017). Hence, H_2SO_4 pretreated biomass was omitted further from studies as the acid pretreatment removed major hemicellulose fraction from biomass. Further evidence for biomass deconstruction by pretreatment was obtained from structural analysis and enzymatic digestibility studies of untreated and autoclaved, steam exploded, NaOH treated and NH_4OH treated biomass.

4.3.6. Structural analysis of PSCB biomass:

Changes in chemical nature and anatomical structure of the biomass due to diverse pretreatments had been studied with Fourier-Transformed Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) respectively.

4.3.6.1. FTIR spectroscopic analysis of SCB biomass:

Specific functional groups and bond patterns present in the structural components of pretreated plants biomass i.e., cellulose, hemicellulose and lignin were analyzed using FTIR technique. Figure 4.7 depicts the comparison of FTIR spectra of raw and pretreated biomass, where the absorption peaks or bands appearing at particular wavenumber(s) are attributes of certain functional groups and chemical bond patterns as explained in Table 3.6 in Chapter 3. In Figure 4.7, The peaks or bands are clustered in two different range of wavenumbers, i.e., 3750-2750 cm^{-1} and 1800-800 cm^{-1} so the peaks are clearly visible in range of 1800-800 cm^{-1} as it covers most wavenumbers attributed to cellulose, hemicellulose and lignin fraction.

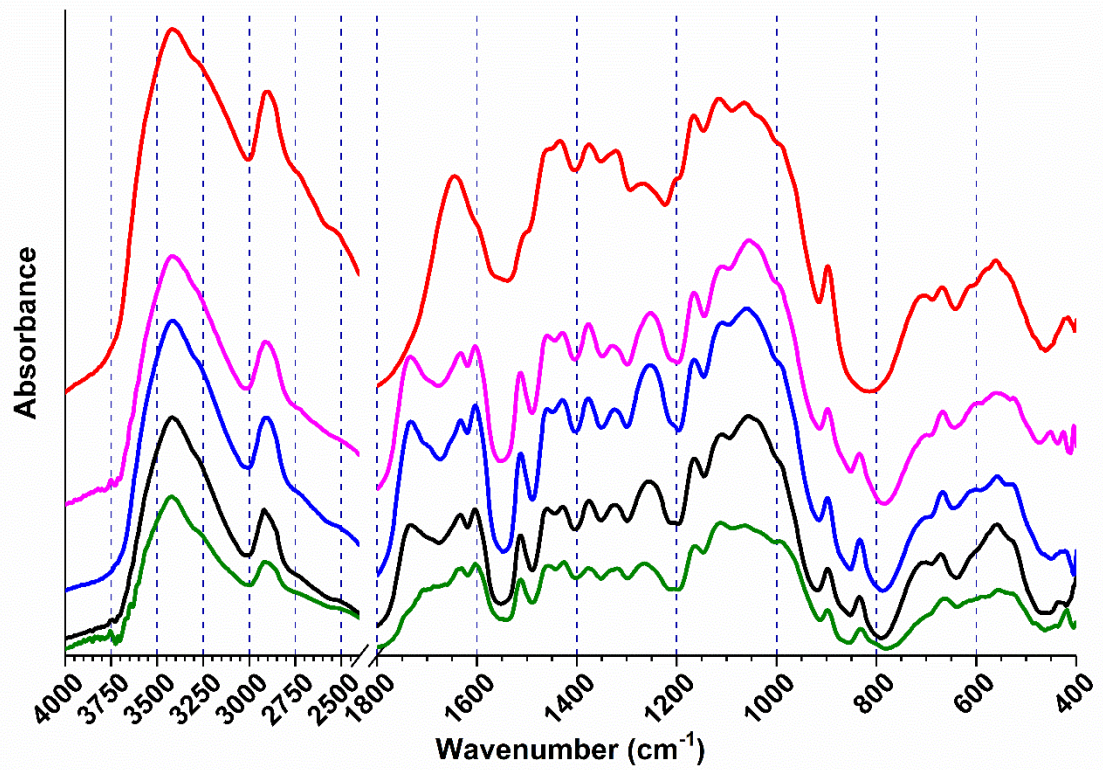


Figure 4.7. FTIR spectra of raw and PSCB biomass fragments:
FTIR spectra of representing chemical changes in untreated (Black, ▬), and pretreated: autoclaved (Blue, ▬), steam-explosion (Pink, ▬), NaOH (Red, ▬) and NH_4OH (Green, ▬) sugarcane bagasse (SCB) biomass solids at wavenumbers of 400 - 4000 cm^{-1}; Y-axis is presented as an offset.

The strong and broad absorbance peak observed at the range of 3450-3300 cm^{-1} and 2950-2820 cm^{-1} were due to stretching exhibited by O-H bond of -OH groups and -C-H stretching respectively. -C-H stretching is attributed to asymmetrical stretching vibration for $-CH_3$, $-CH_2$ or -CH groups. Besides these two major stretches in FTIR spectra, 1800 cm^{-1} to 800 cm^{-1} has been assigned to the major components of the lignocellulosic materials in plant biomass (Kubo and Kadla, 2005; Yang et al., 2011; da Costa Lopes et al., 2013; Garmakhany et al., 2014).

As shown in Figure 4.6, peaks at wavenumbers 1734, 1630, 1600, 1510, 1325, 1270, 1060 and 833 are affected more significantly by NaOH and NH_4OH pretreatments than autoclave and steam explosion pretreatments when compared with raw untreated biomass. Complete disappearance of peak 1734 cm^{-1} suggested efficient breakdown of cross-linkages present between acetyl group of hemicellulose and lignin components which probably led to the separation of the lignin components from the cellulose-hemicellulosic complex matrix (Rajak and Banerjee, 2015). Peaks at 1630,1600, 1510, 1325, 1270, 1070, 1030 cm^{-1} wavenumbers are attributed to chemical bonds and structures present in aromatic lignin components (Kubo and Kadla, 2005;

Rajak and Banerjee, 2015). Thus, decrease in their intensities suggested that the lignin fraction was decreased in NaOH and NH_4OH pretreated biomass. Similar effects of delignification have been observed for AFEX treated rice straw and NaOH treated SCB by Gollapalli et al., (2002) and Utomo et al., (2015) respectively. Peak at 833 cm^{-1} was attributed to out of plane C-H deformations of aromatic ring, decrease and absence of which also pointed towards decrease and absence of aromatic lignin from NH_4OH and NaOH pretreatments (Silva et al., 2017).

1160, 1120 and 897 cm^{-1} are the characteristic absorption bands attributed to carbohydrates like cellulose and hemicelluloses whereas, 1430, 1376, 1320 as well as 1061 cm^{-1} are recognized as characteristic cellulose absorption bands and 1043 as well as 997 cm^{-1} are recognized characteristic bands of hemicelluloses (da Costa Lopes et al., 2013). Presence of peaks at these wavenumber in untreated and all four PSCB biomass suggested that the pretreatment did not drastically alter the cellulose or hemicellulose composition of the SCB biomass (Garmakhany et al., 2014).

The above observations of FTIR spectra concurred with our earlier observations of (i) detection of several peaks of solubles other than xylose and glucose sugar from of NaOH and NH_4OH pretreatment supernatant during HPLC (Section 4.3.3), and (ii) higher loss in dry weight of biomass after NaOH and NH_4OH pretreatments (Section 4.3.4, and 4.3.5). While as discussed earlier, the autoclave and steam explosion treatments seemed to have affected the biomass morphologically more rather than biochemically.

4.3.6.2. Scanning Electron Microscopy of SCB biomass:

Changes in two main features of plant stem anatomy, viz., fibre structure of vascular bundles and parenchymatous medulla or pith were observed in SEM analysis of raw and PSCB. As can be seen from Figure 4.8 (A) Fibre structures (labelled as F) appear to be composed of parallel strips of vascular bundles components and are surrounded by flaky pith cells (labelled as P). The soft and fragile nature of the pith is clearly visible from the micrographs. Further enhanced magnification revealed presence of small pits or pores that connect the neighbouring cells (Figure 4.7 B). Edges of the cell wall of untreated raw SCB appeared smooth and intact, further damage to these cells was indicated with red arrow. Rezende et al., (2011) and Binod et al., (2012) demonstrated similar morphological features of raw SCB without any pretreatment.

When the SCB was subjected to autoclave and steam explosion pretreatments,

the structural morphology of parenchymatous pith region was majorly affected. As can be seen from Figure 4.7 (C-F), the pretreatment enhanced deformations in the cell walls of the pith regions giving it scaly appearance. Extensive damage in edges of pith cell wall were observed in case of steam exploded SCB as compared to autoclaved SCB. At higher magnification the pretreated samples exhibited enhanced roughness on the surface of cell wall as well as unevenness and sharpness of cell wall margins. The conditions used for these two hydrothermal pretreatments affected the biomass with less severity. Similar observations have been reported for SCB (Rocha et al., 2012, 2015). Further, more pronounced structural changes and deconstruction of *Phragmites australis* (common reed), *Miscanthus giganteus*, poplar and wheat straw residues were observed with increased severity of holding conditions under higher pressure (up to 3.4 MPa) and higher temperature up to 160-220 °C during steam explosion pretreatment (Lizasoain et al., 2016; Auxenfans et al., 2017b).

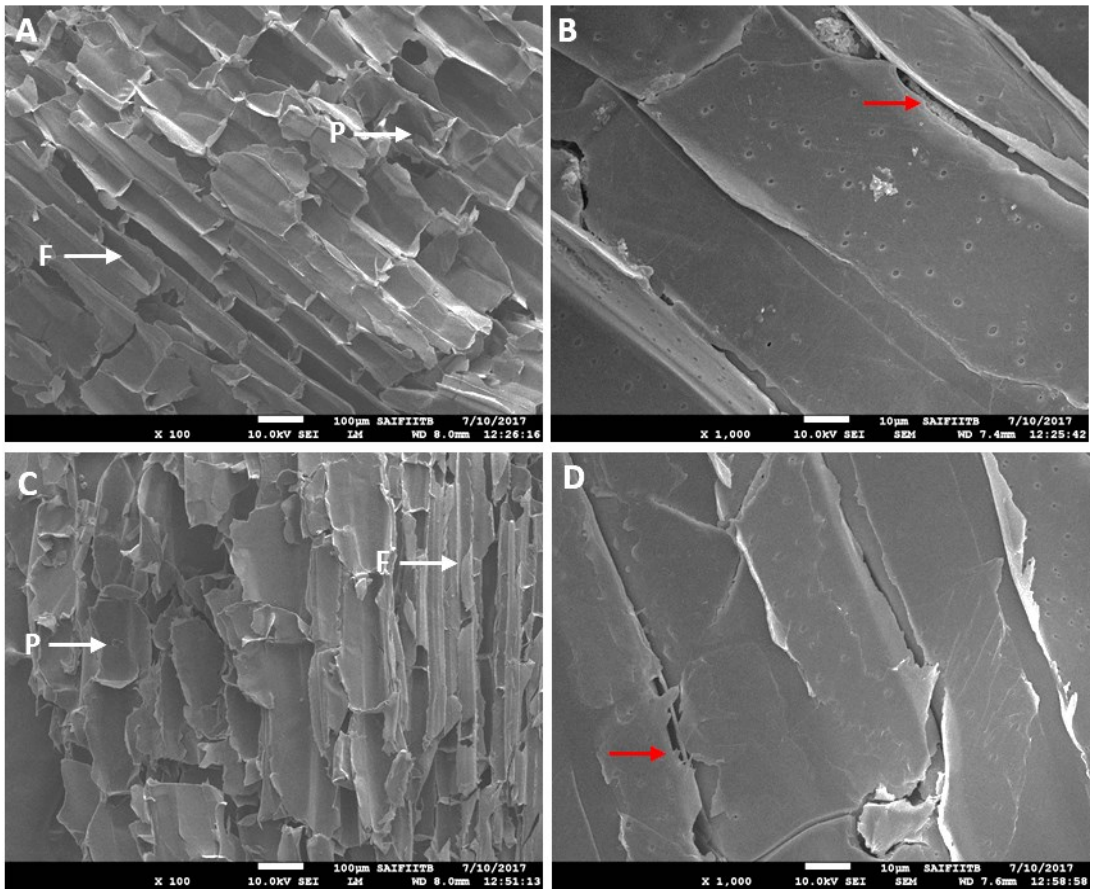
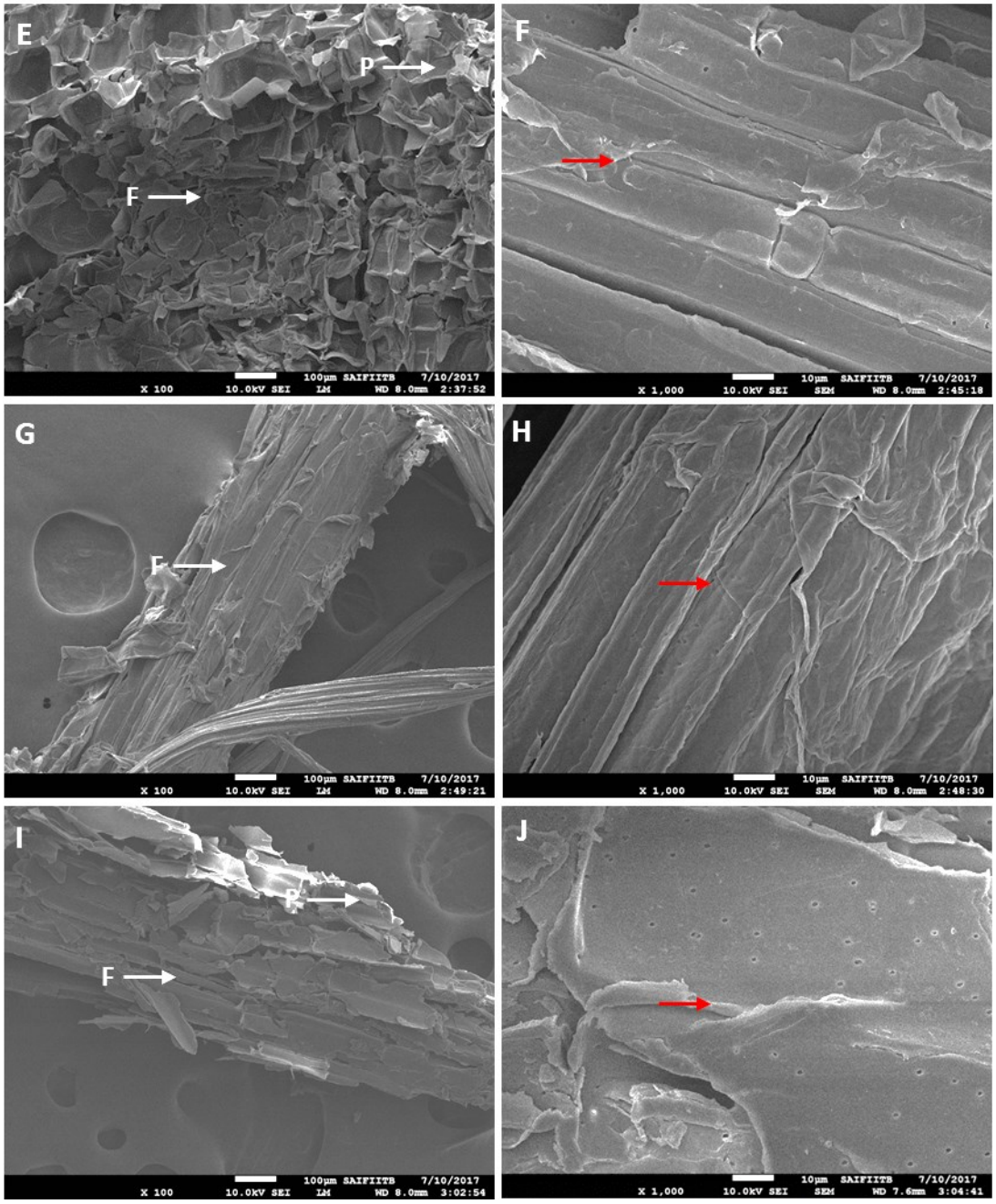


Figure 4.8. Scanning electron micrographs of raw SCB and PSCB biomass fragments: **(A, B)** Raw untreated; **(C, D)** Autoclaved; **(E, F)** Steam Exploded; **(G, H)** NaOH treated; **(I, J)** NH_4OH treated SCB biomass fragments; Images A, C, E, G and I were captured at 100X magnification (white bar represents scale of 100µm); Images B, D, F, H and J were captured at 1000X magnification (white bar represents scale of 10 µm).



NaOH exhibited drastic changes in morphology of PSCB. As presented in Figure 4.7 (G and H), use of NaOH detached the vascular bundles from each other and as a result, separated fibres could be seen. The detached fibre bundles exhibited more curled and twisted appearance suggesting the separation of the medullar pith and vasculature containing cellulosic and hemicellulosic fibres. This might have occurred due to removal of lignin. Also, previous observations of weight loss and disappearance of peaks attributed to lignin from the FTIR spectra in NaOH pretreated SCB can be explained by these observations. Rezende et al., (2011) has reported similar pattern of the fibre separations due to NaOH pretreatment which increased with the enhanced

concentration of NaOH. Pretreatment with diluted liquid ammonia (NH_4OH) also enhanced scaly appearance of the pith regions and under higher magnification, the enhanced roughness of edge is visible. Cao and Aita, (2013) reported similar results for mild ammonia treated sugarcane bagasse.

Thus, morphological, biochemical and structural analysis of PSCB suggested that the biomass has undergone several changes which can be assessed by studies regarding the accessibility of the biomass polysaccharides to saccharifying enzymes.

4.3.7. Studies on amenability of the pretreated biomass to enzymatic saccharification:

Each of the pretreatment used in present studies demonstrated altered morphology and/or biochemical composition of the biomass. The pretreatment is specifically expected to improve accessibility of the biomass to polysaccharide depolymerising enzymes. Kumar and Wyman, (2009) have shown that besides the physico-chemical properties of enzymes and environment which controls the breakdown of polysaccharides, the biomass glucan and xylan accessibility to these enzymes was also one of the important factors, as it is dependent on pretreatment system. Rollin et al., (2011) had also given importance to the increased accessibility to cellulose than to lignin removal. This necessitates the understanding of extent to which the pretreatments have modified the accessibility of plant cell wall polysaccharide components in this study to the core commercial cellulase and accessory xylanase and pectinase enzymes from each of *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 for saccharification.

4.3.7.1. Accessibility of PSCB cellulose to cellulase for saccharification:

Figure 4.8 represents the % saccharification of raw and pretreated biomass suggesting the effect of pretreatments on biomass cellulose accessibility to commercial cellulase. Significant increase in cellulose saccharification of 12-20 fold after NaOH pretreatment, 4-5 fold after NH_4OH pretreatment and 2-3 fold for hydrothermal pretreatments was observed when compared to raw untreated biomass ($p < 0.001$, significance not marked in figure). The possible reason behind this large enhancement in the cellulase activity might be due to lignin removal during NaOH and NH_4OH pretreatments and lignin relocation during hydrothermal pretreatments (Siqueira et al., 2017). Comparison of increased SCB loading for individual pretreatment showed that saccharification increased for autoclaved and steam exploded SCB while it decreased

for NaOH treated SCB. Whereas for NH_4OH treated biomass, saccharification initially increased and then decreased with gradual increase of SCB loading. This difference of saccharification between 1.25 and 2.5% SCB loading as well as between 2.5 and 5.0% SCB loading was more significant in case of only NaOH and NH_4OH treatment among all ($p < 0.001$). NaOH pretreatment was more efficient at lower substrate and chemical loading as the pretreated biomass exhibited more cellulose saccharification.

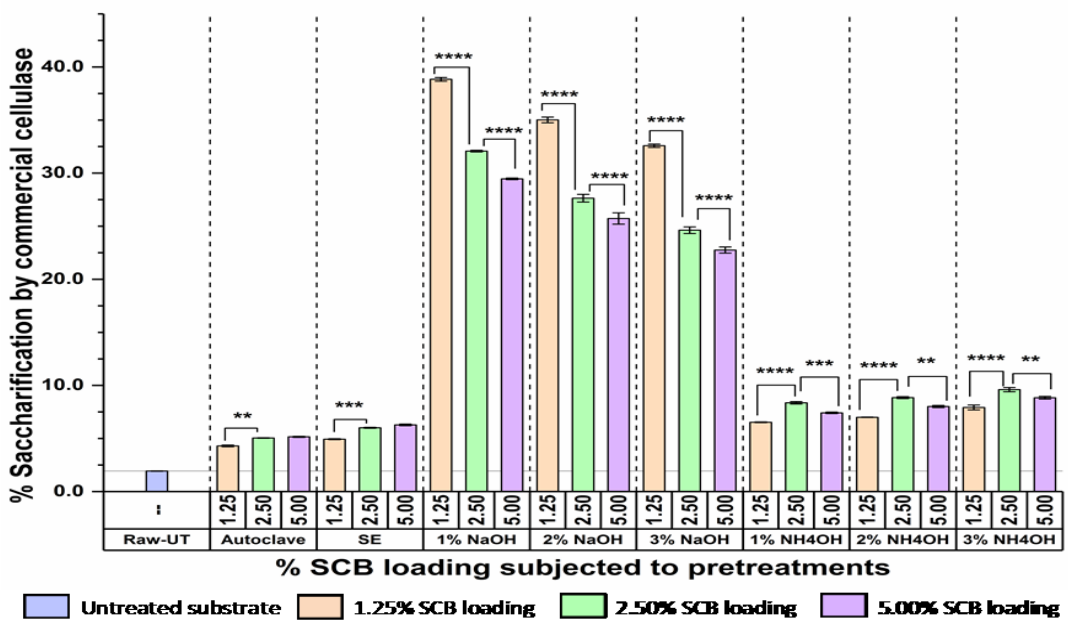


Figure 4.9. Digestibility of cellulose from raw and PSCB by commercial cellulase:
% Saccharification of raw and PSCB exhibited by commercial cellulase.; Significance difference given as * = $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** = $p < 0.0001$); Columns and Error bars represents Mean and Standard Error of Mean (SEM) respectively for n=3.

When Rezende et al., (2011) used 2.5% SCB samples (pretreated with 1% H_2SO_4 followed by different 0.5-5.0% NaOH) with loading of 25 FP units of Accellerase 1500, 73% cellulose digestibility was observed after 48h, whereas in present studies use of only ~3.0 FP units on 2% SCB loadings pretreated with NaOH at different biomass and PSCB loadings, exhibited 25-40% cellulose digestibility in terms of saccharification after 60 h. Several other comparable reports have been published by other investigators using commercial cellulase Cellic® Ctec3 on steam exploded SCB (Silva et al., 2017) and using mixture of Spezyme CP and Novozyme 188, containing 30 units of each, on ammonia treated SCB.

4.3.7.2. Accessibility of pretreated biomass hemicellulose to M35, R31 and J208 xylanases for saccharification:

Figure 4.10 represents the % saccharification of raw and pretreated biomass demonstrating increased hemicellulose accessibility to M35, R31 and J208 xylanases.

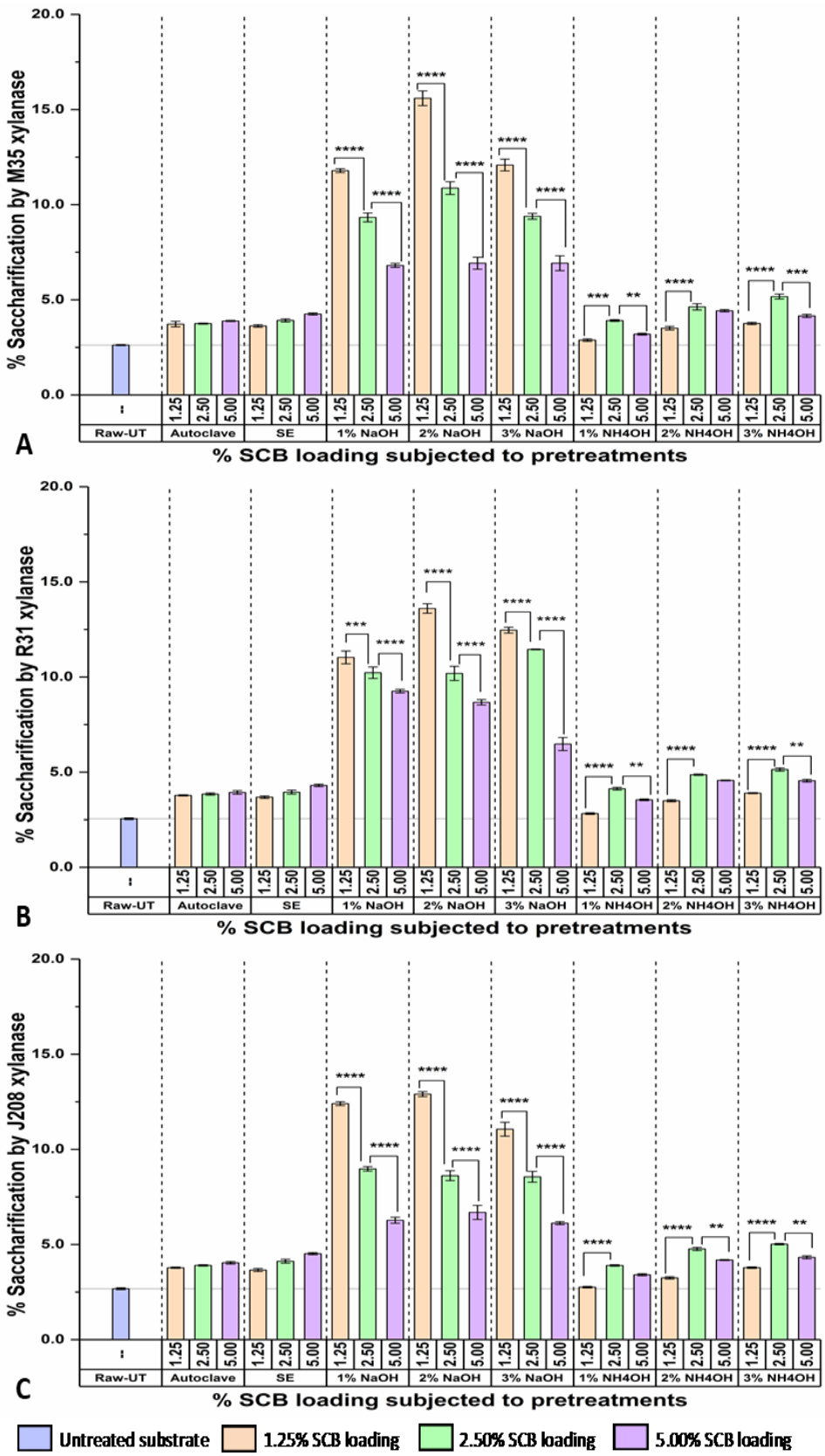


Figure 4.10. Digestibility of xylan from raw and PSCB by crude xylanases:
(A-C) % Saccharification of raw and PSCB by crude xylanase enzymes obtained from **(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Significance difference given as * = $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** = $p < 0.0001$; Columns and Error bars represents Mean and Standard Error of Mean (SEM) respectively for n=3.

Since the hemicellulose fraction in SCB composition is nearly half in comparison to cellulose, the extent of saccharification due to xylanase was less than cellulase. The trend in saccharification for all the three xylanases was same as described ahead. Increased saccharification of PSCB by individual xylanases was observed in comparison to raw SCB which was significant for all pretreatments except 1% NH_4OH pretreatment at 1.25% SCB loading ($p < 0.01$, significance not marked in figure). The saccharification was 2.5 to 6 fold higher for NaOH pretreated SCB at lower substrate loading and maximum at 2% NaOH pretreatment for all the three xylanases. Comparison of increased S/L ratio for individual pretreatment revealed that the saccharification increased for autoclaved and steam exploded SCB and decreased for NaOH treated SCB. Whereas for NH_4OH treated SCB, saccharification initially increased and then decreased with gradual increase of SCB loading. This difference of saccharification between 1.25 and 2.5% SCB loading as well as 2.5 and 5.0% SCB loading was more significant for NaOH and NH_4OH treatment than others ($p < 0.001$).

4.3.7.3. Accessibility of pretreated biomass hemicellulose to M35, R31 and J208 pectinase for saccharification:

Figure 4.11 represents the % saccharification of raw and pretreated biomass demonstrating increased hemicellulose accessibility to M35, R31 and J208 pectinases. Since the galactan components are very less in SCB composition, the saccharification due to pectinase was even less than cellulase as well as xylanase. All the three pectinases exhibited same trend in saccharification as described ahead. Increased saccharification of PSCB by individual pectinase was observed in comparison to raw substrate but this increase was 2-6 fold higher and significant at 1.25% and 2.5% SCB loading during all three NaOH pretreatments for all three pectinases ($p < 0.01$, significance not marked in figure). Saccharification of each pretreated SCB by individual pectinase had increased when compared with raw substrate but this increase was 2 to 6 fold higher and significant for 1.25 and 2.5% SCB loading during each NaOH pretreatment for all three pectinases ($p < 0.01$, significance not marked in figure). During comparison of increased SCB loading for individual pretreatment, saccharification decreased for NaOH treated SCB and increased for autoclaved and steam exploded SCB. While for NH_4OH PSCB, the saccharification initially increased and then decreased with increased SCB loading. This difference of saccharification between 1.25 and 2.5% SCB as well as 2.5 and 5.0% SCB loading was more significant in case of NaOH treatment than others ($p < 0.01$).

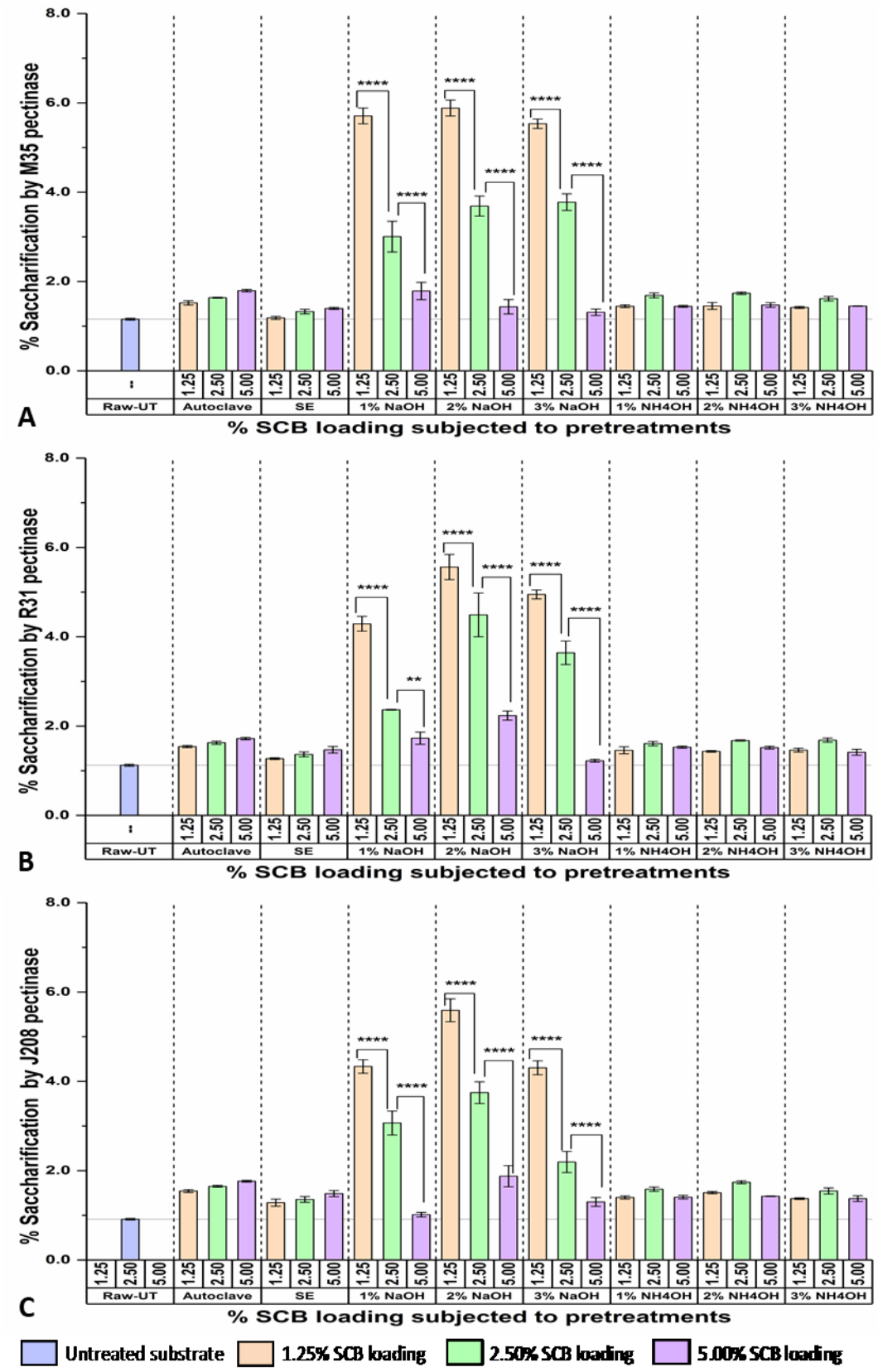


Figure 4.11. Digestibility of pectin from raw and PSCB by crude pectinases:
(A-C) % Saccharification of raw and PSCB by crude pectinase enzymes obtained from **(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Significance difference given as ** ($p < 0.05$), *** ($p < 0.01$) and *** ($p < 0.001$); Columns and Error bars represents Mean and Standard Error of Mean (SEM) respectively for n=3.

Thus, the polysaccharide accessibility for cellulase. Pectinase and xylanase is summarized in Table 4.3 as mentioned below, suggesting the NaOH enhances the polysaccharide accessibility maximum.

Table 4.3. % Saccharification observed from PSCB by individual cellulase, xylanase or pectinase enzymes:

Pretreatment of SCB	% Saccharification observed by		
	Cellulase	Xylanase	Pectinase
Raw	1.93	2.55-2.67	0.91-1.15
Autoclaved	4.30-5.15	3.77-4.02	1.54-1.79
Steam Exploded	4.93-6.28	3.62-4.51	1.18-1.48
NaOH	**22.75-38.83**	**6.1-15.42**	**1.01-5.88**
NH_4OH	6.53-9.60	2.75-5.17	1.37-1.74

Subramaniyan and Prema, (2000) and Jayani et al., (2005) have included exhaustive information on xylanase and pectinase respectively regarding their production, characterization and wide range of applications. Studies on saccharification of polysaccharide containing biomass have been bifurcated into two kinds. In one kind of studies commercial enzyme preparations have been used while in the other kind of studies crude enzymes from microbial source have been reported. In extensive studies on enzymatic saccharification of polysaccharides from different PSCB at 2% SCB biomass loading with individual loading of 6 mg of commercial cellulase (1:4 mixture of Celluclast 1.5L and Novozyme 188), endo-xylanase and pectinase per g of biomass, Li et al., (2014) reported the amount of sugars released by pectinase, xylanase and cellulase was in increasing order. Further, NaOH, steam explosion, H_2SO_4 and H_2O_2 was the order in which the saccharification decreased. These observations are in concurrence with the present studies. The results obtained here also corroborated with earlier observations of Section 4.3.3 and 4.3.4 and suggested that the removal of lignin from middle lamella during NaOH pretreatment is the possible reason for shattered appearance of sugarcane stem which freed the cells and enhanced area of free surfaces to increase the accessibility of cellulose and hemicelluloses to their respective saccharifying enzymes (Rajak and Banerjee, 2016).

Studies where crude enymes are applied for biomass saccharification have been focussed since decades. Microorganisms producing xylanase and pectinase to some extent have been reported for their application in saccharification. Few of these reports are listed below in Table 4.4.

Table 4.4. Biomass saccharification by xylanase and pectinase from bacterial isolates:

Enzyme producing organisms	Biomass and pretreatment	Enzyme (Units/ml)	% Saccharification	References
Bacillus safensis LBF-002	Untreated Rice Straw IR-64,	Crude xylanase (~2.28)	0.5g/L	Lateef et al., (2015)
Bacillus altitudinis DHN 8	Sorghum straw, untreated, acid, alkali, alkali peroxide	Crude xylanase (~70)	0.4% (untreated), 2.0% (acid treated), 2.5% (alkali treated), 3.5% (alkali peroxide)	Adhyaru et al., (2014)
Bacillus amyloliquefaciens XR44A	Brewer's spent grain,	Partially purified xylanase (~10.5)	~43%	Amore et al., (2015)
Bacillus sp. CX6	Wheat strw	Crude xylanase (~700)	~6%	Ahmed et al., (2018)
Bacillus safensis M35	NaOH PSCB	Crude xylanase (4.8)	15.6%	This study
Bacillus altitudinis R31	NaOH PSCB	Crude xylanase (5.0)	13.6%	This study
Bacillus altitudinis J208	NaOH PSCB	Crude xylanase (5.5)	12.9%	This study
Bacillus safensis M35	NaOH PSCB	Crude pectinase (11.3)	5.8 %	This study
Bacillus altitudinis R31	NaOH PSCB	Crude pectinase (14.6)	5.7 %	This study
Bacillus altitudinis J208	NaOH PSCB	Crude pectinase (14.8)	5.6 %	This study

Table 4.4 presents comparative of reported values for enzyme units and saccharification. The crude xylanases and pectinases from the isolates in present study gave 12.9-15.6 % and 5.6-5.8% saccharification from SCB respectively, which is better than most of the reports except *Bacillus amyloliquefaciens* XR44A gave higher saccharification of ~43% from Brewer's spent grain than our isolates, but with partially purified enzyme after ammonium sulphate precipitation. Though, *Bacillus* sp. CX6 has been reported to produce ~700IU of xylanase but it exhibited saccharification of only ~6% on Wheat straw. On other hand in present studies, ~5.0 units of xylanase produced higher saccharification of 12.9-15.6% on NaOH PSCB.

4.3.7.4 Brightfield and fluorescent microscopic studies on cellulase mediated changes in cell wall anatomy of SCB biomass:

Action of cellulase on the cellulosic components of SCB biomass after pretreatment was also studied microscopically to visualise anatomical changes. Figures

4.12- 4.16 represent the images of transverse sections of stele of sugarcane stem comprising of xylem-phloem containing vascular bundles embedded in medullar pith of parenchymatous tissue. On safranin staining, the DW treated cell wall (control) appeared red under light microscopy (Figure 4.12 A, D). In fluorescence microscopy, cellulose component of the cell wall gave green fluorescence (Figure 4.12 B, E) whereas lignin component gave red fluorescence (Figure 4.12 C, F). Sant'Anna et al., (2013) has reported similar observations for safranin stained sugarcane. As can be seen from light and fluorescence microscopic images in the DW treated control (Figure 4.12), no difference in region of pith and vascular bundles was observed even after 120 min of incubation with DW. As can be observed the cellulose and lignin are closely associated in the cell wall.

In figures 4.13 to 4.16 cellulase treated transverse sections of pretreated sugarcane stele are shown where the cell wall gives discontinuous appearance in brightfield micrographs while intensity of green fluorescence is lost or reduced in the fluorescence micrographs due to digestion of cellulose in the parenchymatous pith tissue. Similarly, the region of vascular bundle containing sieve plates and companion cells, the two major components of phloem vasculature, also exhibited disappearance of green fluorescence. Whereas, the xylem components and the sclerenchyma sheath of the vascular bundle exhibiting red fluorescence attributed to lignin remained intact. Similar observations have been reported by Sant'Anna et al., (2013). When observations were correlated with the anatomy of the sugarcane bagasse as explained in chapter 1, the disappearance of the green fluorescence from the field and appearance of the discontinuous cell wall instead of intact ones suggested that cellulose disruption or breakdown had taken place. Cellulase action on untreated sections revealed similar observations, however the intact layers of pith tissue surrounding the vascular bundles were noticeable (Figure 4.13). This can be clearly attributed to lack accessibility of cellulose to the cellulase.

In sections of autoclaved and steam exploded biomass (Figures 4.14 & 4.15) although, disruption of cellulosic cell wall was commonly observed, the inner layers of parenchymatous cells surrounding the vascular bundles were also disrupted and the extent of damage increased as the incubation time increased. The damage to pith tissue in case of steam explosion was higher as compared to autoclave treatment (Figure 4.14D & Figure 4.15D). These and the cellulase mediated saccharification results (Section 4.3.7.1) suggested that the steam explosion and autoclave pretreatment with

lower severity might not have removed the lignin but might have affected partial distribution of lignin which have enhanced the cellulose accessibility and which in turn enhanced the saccharification.

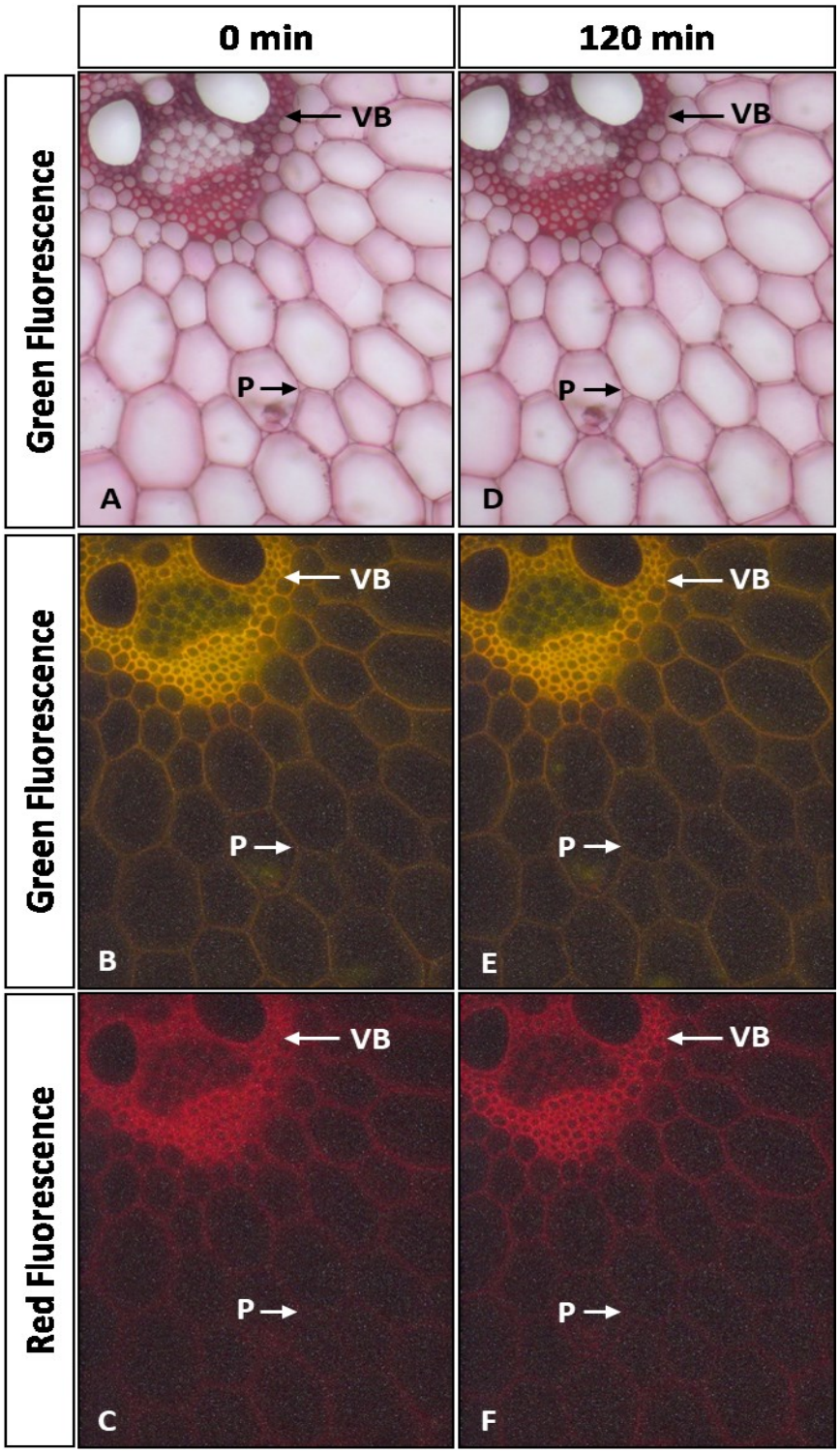


Figure 4.12. Microscopy of DW treated sugarcane pith section depicting stained cell wall: Images **A**, **B** & **C** captured at 0 min and images **D**, **E** & **F** captured after 120 min of incubation with buffer; Brightfield microscopic images (**A** & **D**): Fluorescence microscopic images showing green fluorescent signal for distribution of cellulose (**B** & **E**) and red fluorescent signal for distribution of lignin (**C** & **F**).

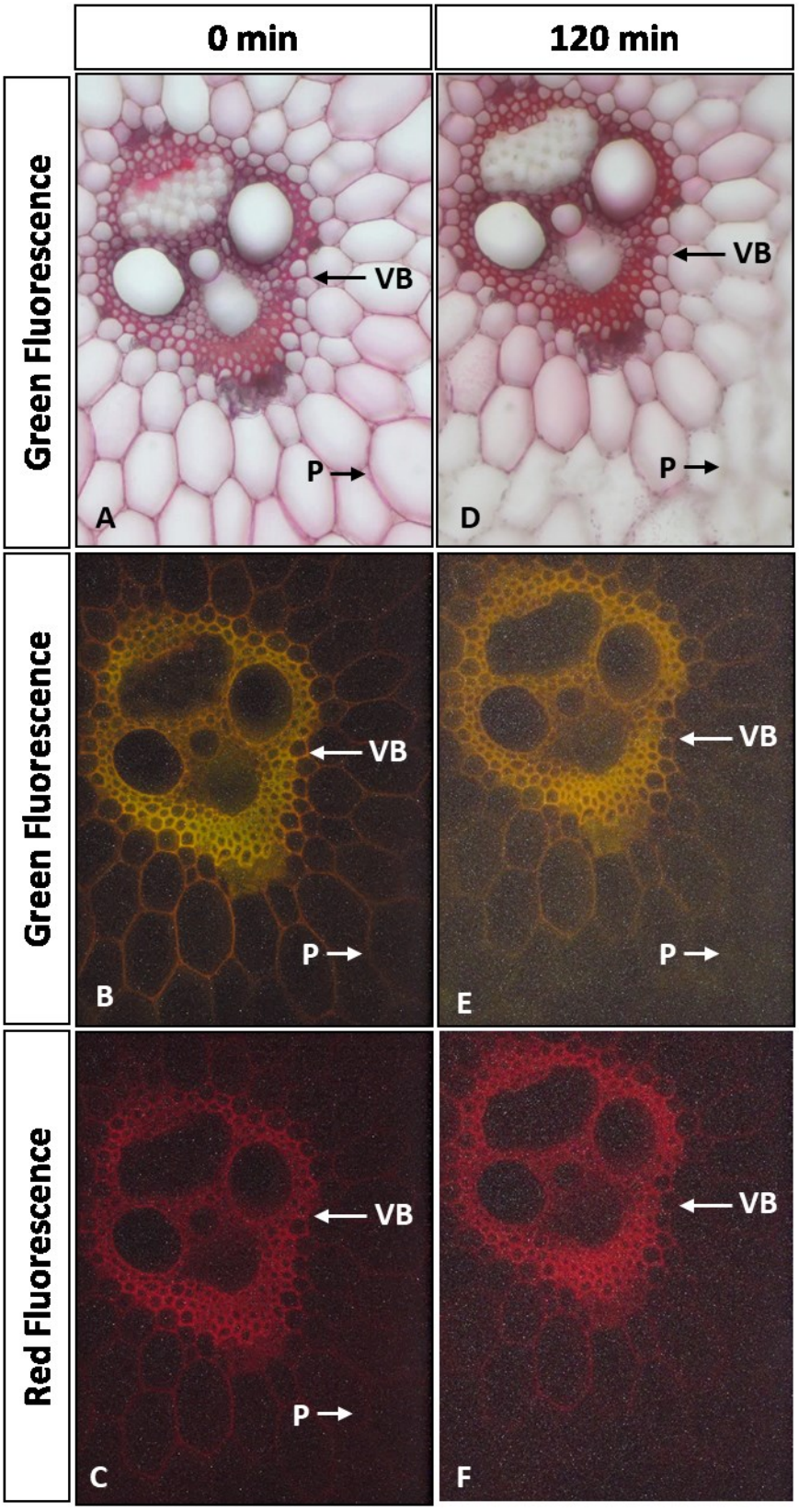


Figure 4.13. Microscopy of safranin stained raw (untreated) sugarcane transverse section depicting stained cell wall after incubation with cellulase:
Images **A**, **B** & **C** captured at 0 min and images **D**, **E** & **F** captured after 120 min of incubation with commercial cellulase; Brightfield microscopic images (**A** & **D**): Fluorescence microscopic images showing green fluorescent signal for distribution of cellulose (**B** & **E**) and red fluorescent signal for distribution of lignin (**C** & **F**)

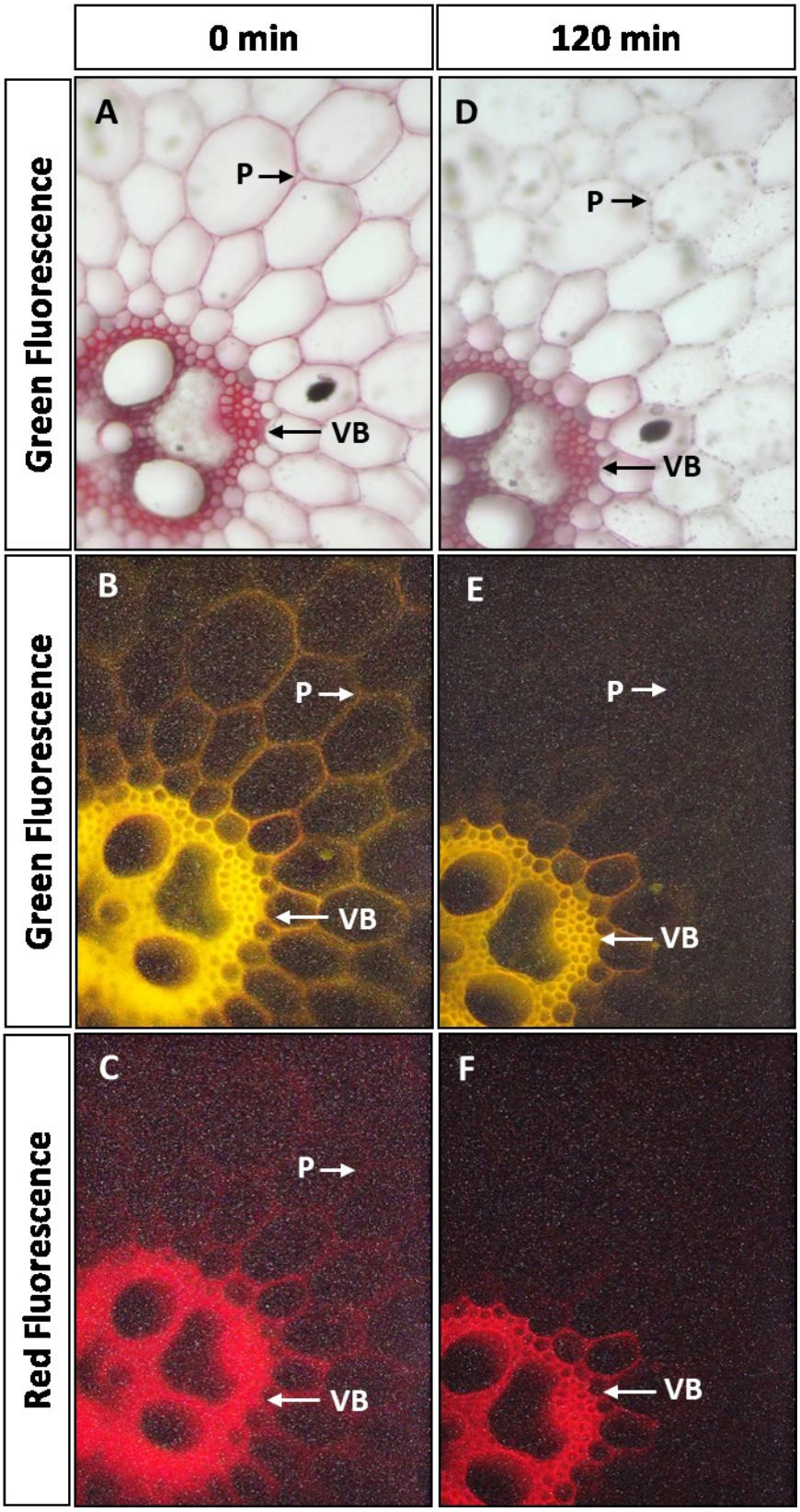


Figure 4.14. Microscopy of safranin stained autoclaved sugarcane cell wall before and after incubation with cellulase:
Images **A**, **B** & **C** captured at 0 min and images **D**, **E** & **F** captured after 120 min of incubation with commercial cellulase; Brightfield microscopic images (**A** & **D**): Fluorescence microscopic images showing green fluorescent signal for distribution of cellulose (**B** & **E**) and red fluorescent signal for distribution of lignin (**C** & **F**)

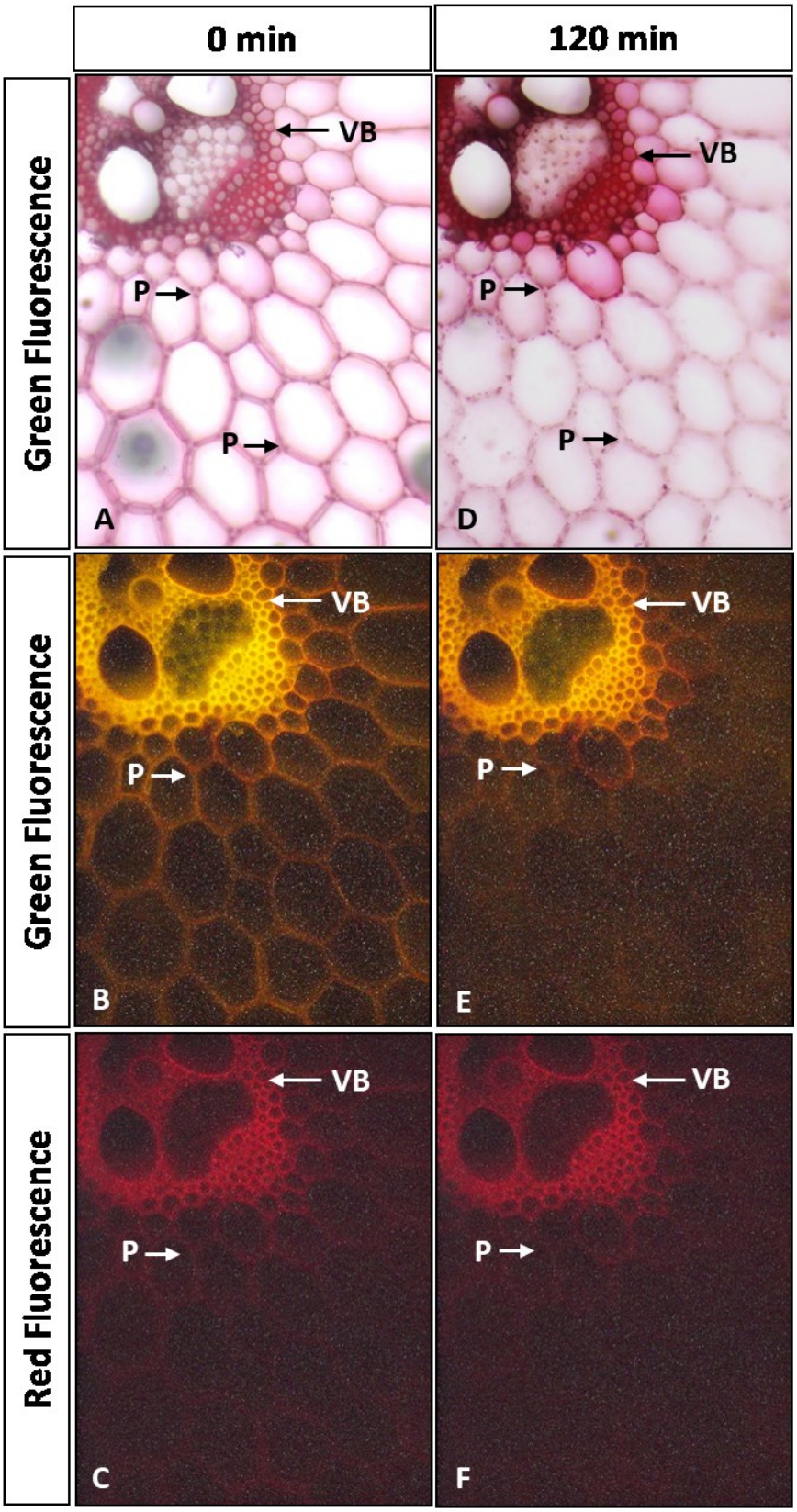


Figure 4.15. Microscopy of safranin stained steam exploded sugarcane cell wall before and after incubation with cellulase:
Images **A**, **B** & **C** captured at 0 min and images **D**, **E** & **F** captured after 120 min of incubation with commercial cellulase; Brightfield microscopic images (**A** & **D**): Fluorescence microscopic images showing green fluorescent signal for distribution of cellulose (**B** & **E**) and red fluorescent signal for distribution of lignin (**C** & **F**).

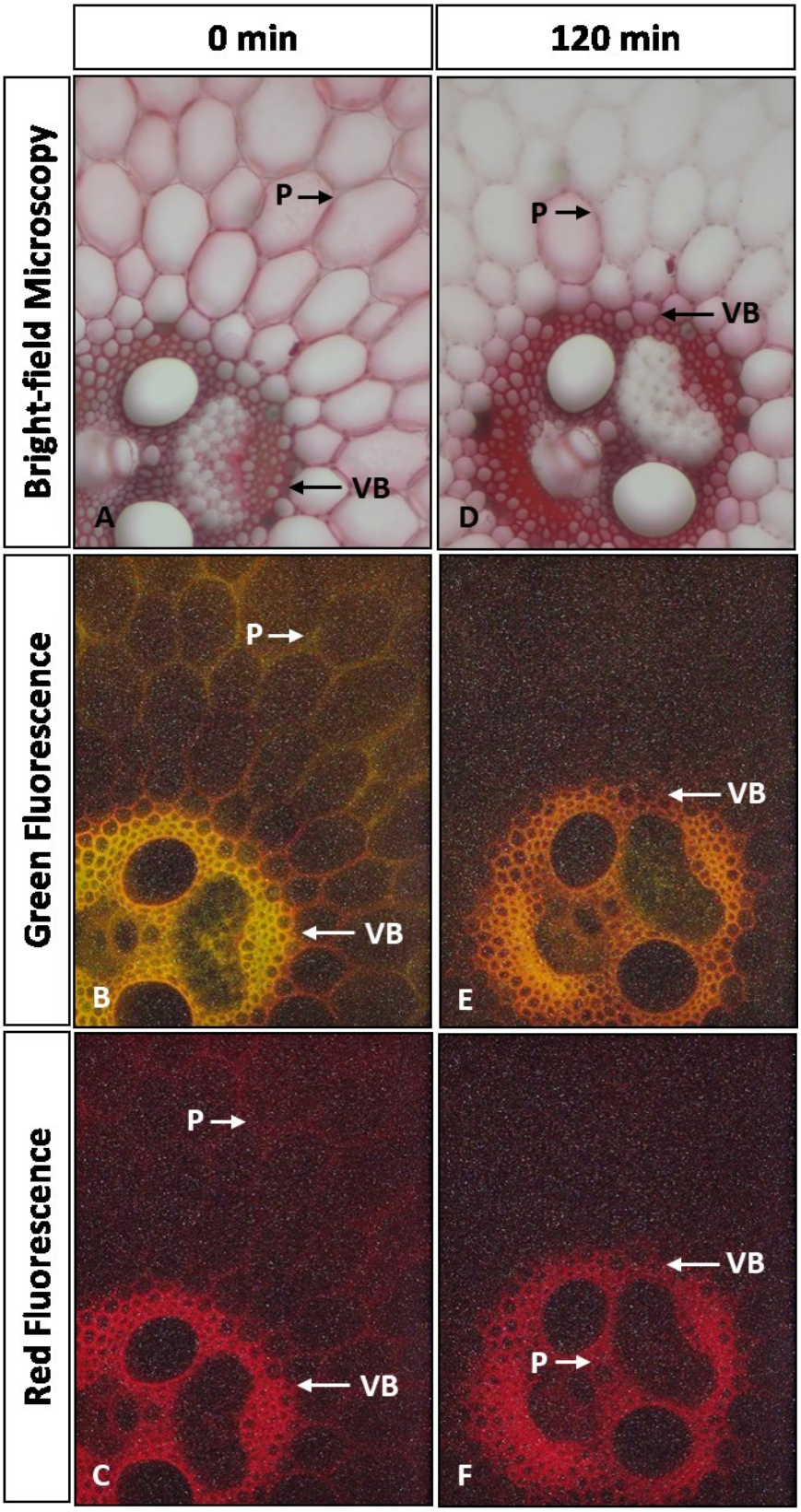


Figure 4.16. Microscopy of safranin stained NH_4OH sugarcane cell wall before and after incubation with cellulase:
Images **A**, **B** & **C** captured at 0 min and images **D**, **E** & **F** captured after 120 min of incubation with commercial cellulase; Brightfield microscopic images (**A** & **D**): Fluorescence microscopic images showing green fluorescent signal for distribution of cellulose (**B** & **E**) and red fluorescent signal for distribution of lignin (**C** & **F**)

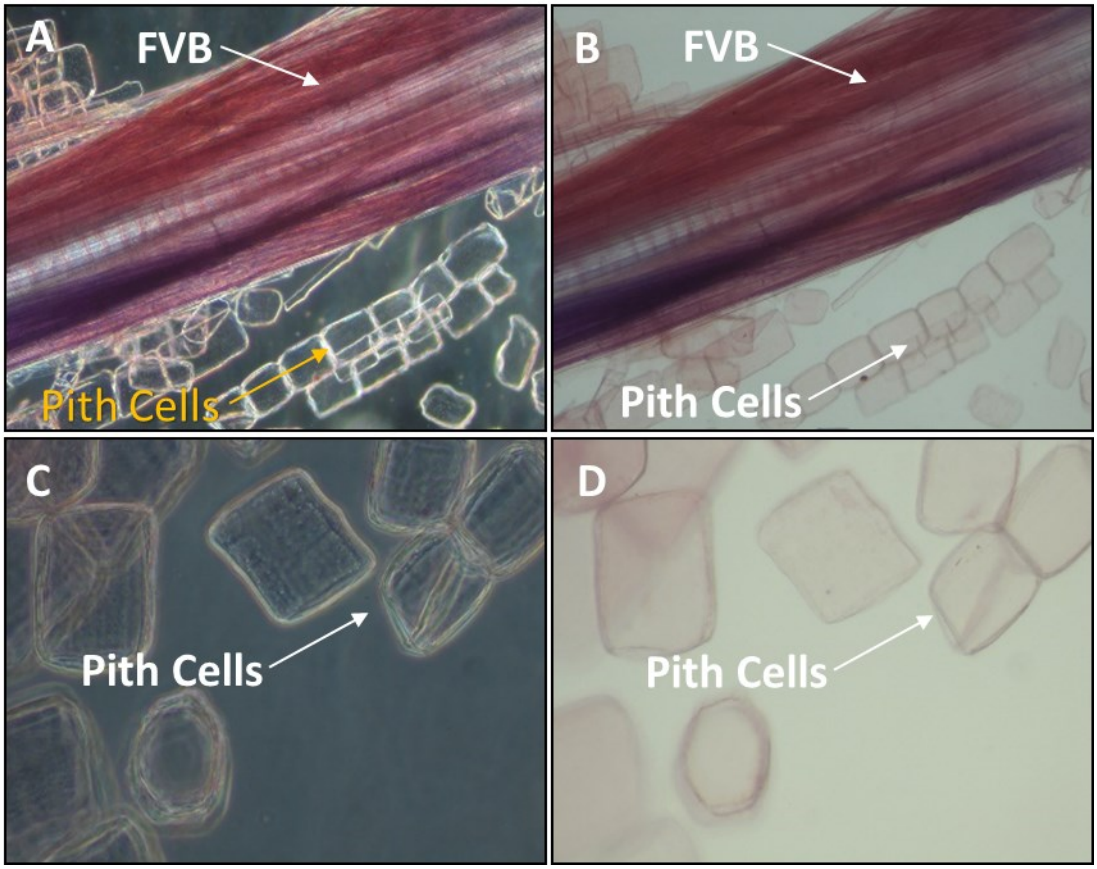


Figure 4.17. Microscopy of safranin stained NaOH treated sugarcane cell wall:
Images **A** & **B** represents the fibrous vascular bundle (labelled as **FVB**) and the fragments of surrounding parenchymatous pith cells; Images **C** & **D** represents the individual cells of pith parenchyma separated after NaOH pretreatments; **A** & **C** are dark field microscopy images; **B** & **D** are brightfield microscopy images.

In figure 4.16, sections of NH_4OH treated stele depicted digestion of cellulosic cell wall and reduction in green fluorescence intensity, the inner layers of pith cells were equally affected as by autoclave and steam explosion treated biomass suggesting that the AFEX treatment renders the lignin distribution by partial removal of lignin from cell wall matrix making the cellulose accessible.

As shown in Figure 4.17, the stele of sugarcane biomass was shattered into fibers by NaOH treatment. So, the sectioning of pretreated biomass was not possible. Examination of fibrous filaments under light microscopy revealed that the parenchymatous pith region had completely disintegrated into smaller fragments leaving vascular bundles apart and could not be observed properly under fluorescence microscopy. Middle lamellae and cell corners were also reported to harbour noticeable amount of lignin in completely grown cells (Bond et al., 2008; Sant'Anna et al., 2013). The possible reason behind the shattering of the biomass might be the removal of the solubles other than sugars from the biomass due to NaOH pretreatment. This might

include lignin, distributed at middle lamellae and cell corners and as an effect of its removal, the vascular bundles and pith parenchyma has separated apart.

Thus, although pretreatments have enhanced cellulose accessibility of the SCB biomass to cellulase, NaOH treatment gave the best results. The accessibility of cellulse in terms of enzymatic saccharification can be further improved by applying enzyme mixtures or cocktails. To improve the yield of fermentable sugars these pretreated biomass were further subjected to saccharification by enzyme cocktails of commercial cellulase and crude xylanases and pectinases from present study.