Chapter 5

Enzyme cocktail mediated saccharification of pretreated sugarcane bagasse (PSCB)

5.1. Introduction:

Plant cell wall is a complex matrix comprising polysaccharides like cellulose, xylan, mannan and pectin (rhamnogalacturonan). Efficient breakdown of such plant cell wall matrix to their oligo or monomeric components requires different enzymes to work together on these polysaccharides. SCB comprises of 41.4, 28.2 and 1.3 % of cellulose, hemicellulose (Xylan polymers) and galactan (pectic polymers) respectively (Ferreira-Leitão et al., 2010). Breakdown of such polysaccahrides to oligo- or monomeric units invariably requires the action of core cellulases assisted by accessory xylano-pectinolytic enzymes. Thus, the saccharification is usually dependent upon the synergistic action of each enzyme (Banerjee et al., 2010c).

As explained in Chapter 3, Section 3.1, "Core" enzymes (e.g., endoglucanase, β-1,4-glucosidase, cellobiohydrolases, etc.,) carry out saccharification of major polysaccharide component from plant cell wall, i.e., cellulose and control the rate of saccharification process yielding glucose. To enhance the rate of cellulose saccharification, the "accessory" group of enzymes are required which remove the hemicellulose covering and free the cellulose fibres, enhancing their accessibility and digestibility by core cellulase (Banerjee et al., 2010c). Thus, an enzyme cocktail of core and accessory group of enzymes can be formulated and applied to enhance the saccharification process.

As elaborately explained in Chapter 1, Section 1.9, the synergism can be studied using either additive approach or substitutive approach. In additive studies, one enzyme loading is kept constant in a cocktail throughout and effect of addition of different loadings of other enzymes are analyzed. While in substitutive approach the part of one enzyme loading is substituted or replaced with the same load of other enzymes and effects are analyzed. In literature both the approaches have been used to study the synergistic saccharification (Kumar and Wyman, 2009b; Hu et al., 2011; Kostylev and Wilson, 2012; Zhang et al., 2013). Van Dyk and Pletschke, (2012) has reviewed application of diverse commercial cellulases like Celuclast 1.5L, Multifect® CL, Spezyme CP, Novozyme 188, Accelrase, Cellic CTec3 for plant biomass saccharification either alone or with assistance of either commercial accessory enzymes like Multifect pectinase, Multifect xylanse, Accellerase xylanase etc., or accessory enzyme produced by certain laboratory strains of bacteria or fungi (Varga et al., 2002; Ferrer et al., 2005; Berlin et al., 2007; Yoshida et al., 2008; da Costa Lopes et al., 2013;

Vandenbossche et al., 2014;, Li et al., 2014a; Rollini et al., 2014; Agrawal et al., 2015; Maitan-Alfenas et al., 2015a; Rocha et al., 2015; Siqueira et al., 2017; Banerjee et al., 2017; Dutra et al., 2017). Commercial cellulases after supplementation with Multifect Xylanase and Multifect Pectinase have been reported to show enhancement in glucan conversion of the dilute acid pretreated corn stover (Berlin et al., 2007) and ammonia fibre expansion (AFEX) treated rice straw (Zhong et al., 2009). Improved saccharification in case of WB and SCB has been obtained from the enzyme cocktail containing cellulase, pectinase and xylanase enzymes produced by fungus *Chrysoporthe cubensis* in comparison to commercial enzymes (Maitan-Alfenas et al., 2015a; Dutra et al., 2017).

As seen in Chapter 4, Section 4.3.7.1, only 38.8% saccharification was achieceved by core cellulase alone, which suggested that, there are possibilities for improvement of saccharification, if further cocktail formulation of core cellulase and accessory xylanase-pectinase enzymes are used to study the saccharification. Hence, in present chapter, xylanases and pectinases from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were used as accessory enzymes to core commercial cellulase Primafast®200 and their effect in additive as well as substitutive synergism have been studied.

5.2. Materials and Methods:

5.2.1. 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) is a raw industrial waste biomass product left over after squeezing the juice out of sugarcane for sugar processing. SCB was ground, washed, dried and subjected to different pretreatments i.e., Autoclave, Steam explosion, Alkali, AFEX and Acid. This pretreated SCB biomass was used for enzyme cocktail mediated saccharification studies and referred to as PSCB.

Citrus Peels (CP) and Wheat Bran (WB) were collected, processed and stored as mentioned in Chapter 2, Section 2.2.1,and were used as a crude polysaccharide substrate in media for selective production of crude xylanase and pectinase enzymes. They will be referred as WB and CP throughout the chapter.

Three selected isolates *Bacillus safensis* M35, *Bacillus altitudinis* R31 and

Bacillus altitudinis J208 obtained from camel, bull and buffalo dung respectively were maintained and stored on NA plate at 4-6 ºC. Xylanases and pectinases produced by these isolates were used as accessory enzymes for enzymatic digestion of pretreated biomass studies presented in this chapter. Primafast®200 (Genencor, Du-Pont) was properly diluted and used as source of commercial cellulase for saccharification studies in cocktails and is referred to as commercial cellulase in the text.

5.2.2. 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, Chapter 3. The 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 these studies. Primafast®200 will be referred to as commercial cellulase as it was a commercial source of cellulase. The assay methods for quantification of these enzymes is given in Section 3.3.12 of Chapter 3.

5.2.3. Enzyme cocktail mediated saccharification of PSCB biomass:

2.0% w/v of individual untreated and PSCB biomass substrate was suspended in 10 ml of 50 mM Tris-Cl buffer pH 7.0 for core and/or accessory enzyme cocktail mediated saccharification studies. 100 µg/ml of each, Sodium azide, Ampicillin, Kanamycin and Streptomycin was amended to prevent microbial contamination during the saccharification assay. Biomass saccharification by individual enzymes (C, P and X), cocktail of two types of enzymes (CP, CX and PX), and cocktail of all three types of enzymes together (CPX) was carried out. The practical yield (PY) obtained by saccharification activity of cocktail was compared with the cumulative yield (CY) which is the theoretical sum of saccharification activity of individual enzyme present in that cocktail. 400 µg (in terms of protein) of xylanase (X) and/or 400 µg (in terms of protein) of pectinase (P) with 320 µg (in terms of protein) of commercial cellulase (C) were loaded per 200 mg biomass to formulate enzyme cocktail. As mentioned in Section 5.1, both, additive as well as substitutive approach were used to study the synergistic interaction between xylanase and pectinase enzymes in two different saccharification experiments as explained ahead.

5.2.3.1. Additive saccharification studies with cocktail of commercial cellulase and/or xylanases and/or pectinases from individual isolate:

Core commercial cellulase (C) and accessory pectinases (P) and xylanases (X) obtained from a single source organism i.e. *B. safensis* M35 or *B. altitudinis* R31 or *B. altitudinis* J208, were individually used to formulate different cocktails, i.e., PX, CP, CX as well as CPX and strain name of source organism was prefixed with the cocktail name. The reaction mixture was loaded with 320 µg of commercial cellulase and either 400 µg of pectinase and/or 400 µg of xylanase from one single isolate (M35, R31 or J208 individually) per 200 mg of untreated and PSCB substrate. Addition of accessory pectinase and/or xylanase to the core cellulase was done in an additive way which enhanced the total protein load. Hence, the synergism studies followed the additive approach. Each experimental system was incubated in shaking condition at 160 rpm, 40 °C up to 60 h. After 12 and 60 h of incubation, hydrolysate samples were withdrawn and estimated for released reducing sugars by DNS method.

5.2.3.2. Substitutive saccharification studies with cocktail of commercial cellulase and/or xylanases and/or pectinases from all isolates:

The reaction mixture was loaded with 320 µg (in terms of protein) of commercial cellulase Primafast®200 and 133 µg of xylanase and/or pectinase from each of M35, R31 and J208 to obtain total xylanase or pectinase loading of 400 µg per 200 mg of dry PSCB biomass. Addition of M35, R31 and J208 P, X or PX to the cocktail was done in a substitutive manner maintaining the total protein load constant. These cocktails further will be referred as substitutive cocktails. Each experimental system was incubated in shaking condition at 160 rpm, 40 °C up to 60 h. After 12 and 60 h of incubation, samples were withdrawn and estimated for released reducing sugar by DNS method. A separate experiment with 133 µg of each of M35, R31 and J208 xylanase or pectinase cocktail to obtain total loading of 400 µg of xylanase or pectinase per 200 mg of dry raw SCB biomass was carried out.

5.2.3.3. Estimation of reducing sugar from cocktail hydrolysate and calculation of degree of synergism (DS):

300 µl of di-nitro salicylic acid (DNS) reagent was added to 300 µl of enzyme hydrolysate sample 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 DW to each reaction system and absorbance was measured at 540 nm (adapted and modified, Miller, 1959; Ghose and Bisaria, 1987). Reducing sugar released after

hydrolysis was quantified using D-glucose as standard and % saccharification can be calculated as the amount of reducing sugar in % released after hydrolysis of total substrate provided.

$$\%\ Saccharification = \frac{released\ reducing\ sugar\ (mg/ml)}{Substrate\ used\ (mg/ml)} \times 100 \qquad \text{(Eq. 5.1)}$$

Similarly, synergism can be calculated from the ratio of practically observed % saccharification by enzyme cocktail to cumulative % saccharification due to individual enzymes of cocktail.

$$Degree\ of\ synergism = \frac{\%\ Saccharification\ by\ enzyme\ cocktail}{Cummulative\ \%\ saccharification\ by\ individual\ enzyme} \qquad \text{(Eq. 5.2)}$$

5.2.4. Structural analysis of CPX cocktail treated PSCB biomass:

During saccharification enzymes alter the structural composition of plant cell wall. Scanning Electron Microscopy (SEM) was used for morphological and structural analysis, while Fourier-Transform Infrared spectroscopy (FTIR) was used for chemical analysis of PSCB biomass treated with CPX cocktail for substitutive saccharification as explained in Section 5.2.3.2.

5.2.4.1 Scanning Electron Microscopy (SEM):

The selected PSCB 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, 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. (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 10 kV under FEG-SEM (JEOL, JSM-7600F) at Sophisticated Analytical Instrument Facility (SAIF), IIT-Powai, Mumbai, India. Electron micrographs were taken at desired magnifications.

5.2.4.2. 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 laboratory 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).

5.2.5. Data analysis:

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

5.3. Results and Discussion:

Studies presented in Chapter 3, Section 3.3.2, exhibited efficient synergism between enzymes for raw biomass saccharification and suggested compatibility of xylanase and pectinase obtained from the same isolate with each other as well as with commercial core cellulase to formulate efficient cocktails. Based on this, the additive and synergistic interactions between xylanase and pectinase enzymes were used to formulate different cocktails containing commercial cellulase and/or xylanase-pectinase enzymes for biomass saccharification.

5.3.1. Additive saccharification studies with different cocktails of C, M35, R31 and J208 P and M35, R31 and J208 X enzymes:

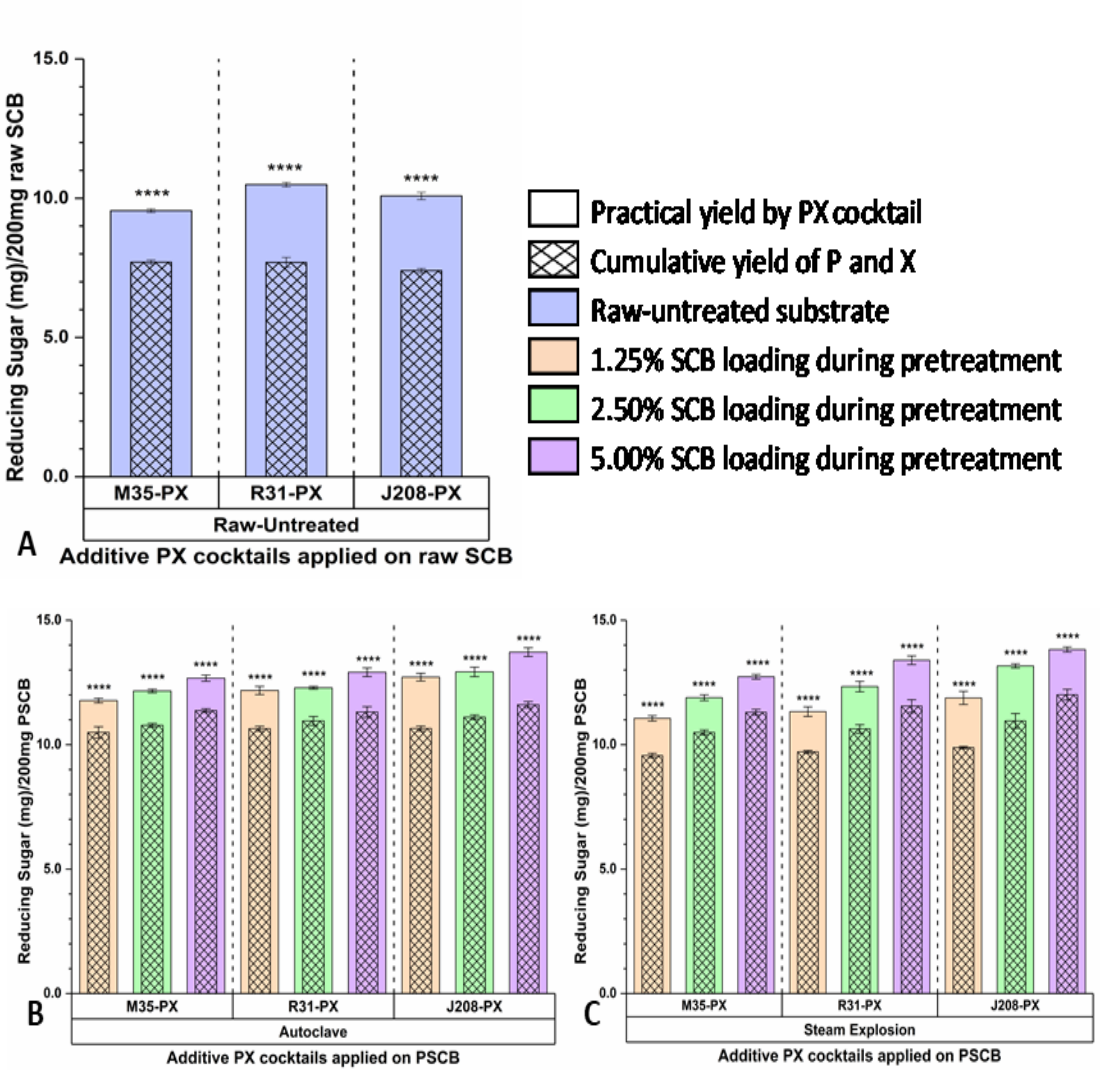
Saccharification studies of PSCB biomass were performed using, pectinase-xylanase (PX), cellulase-xylanase (CX), cellulase-pectinase (CP) and cellulase-pectinase-xylanase (CPX) cocktails up to 60 h. For all of the cocktail combinations, in comparison to 12 h (data not shown here), the yield of saccharification from raw and PSCB biomass enhanced substantially after incubation up to 60 h. The highest saccharification yield was observed in case of NaOH PSCB at least biomass loading of 1.25% which drastically decreased, as the substrate loading increased during each NaOH pretreatment.

5.3.1.1. Saccharification of PSCB biomass by additive PX cocktails:

As can be seen from Figure 5.1 (A-I), positive synergism between P and X for saccharification was observed as the practical yield by PX cocktail from each sample

exceeded the cumulative yield of P and X enzymes. The difference between practical and cumulative values was significant ($p < 0.0001$) for most comparisons.

Three PX cocktails M35-PX, R31-PX and J208-PX practically yielded reducing sugar in the range of ~9.5-10.5 mg from raw SCB against the calculated cumulative yield in the range of ~7.4-7.1 mg (Figure 5.1 A). Enhanced saccharification was observed for each PX cocktail in case of pretreated substrate as compared to raw biomass. For autoclave and steam explosion, the practical yield by PX cocktail was observed in the range of ~11.0-13.8 mg which was higher than their cumulative yield of ~9.5-12.0 mg (Figure 5.1 B, C). NaOH pretreatment exhibited highest improvement in the biomass saccharification as the practical yield by PX cocktail reached in the range of ~17.5-51.0 mg, which was higher than their cumulative yield of ~14.8-42.4 mg (Figure 5.1 D-F). For NH_4OH pretreated biomass, the practical yield of reducing sugar after 60 h of enzymatic saccharification was calculated to be in the range of ~11.7-15.8 mg which was higher than the cumulative yield of ~8.3-13.6 mg (Figure 5.1 G-I).



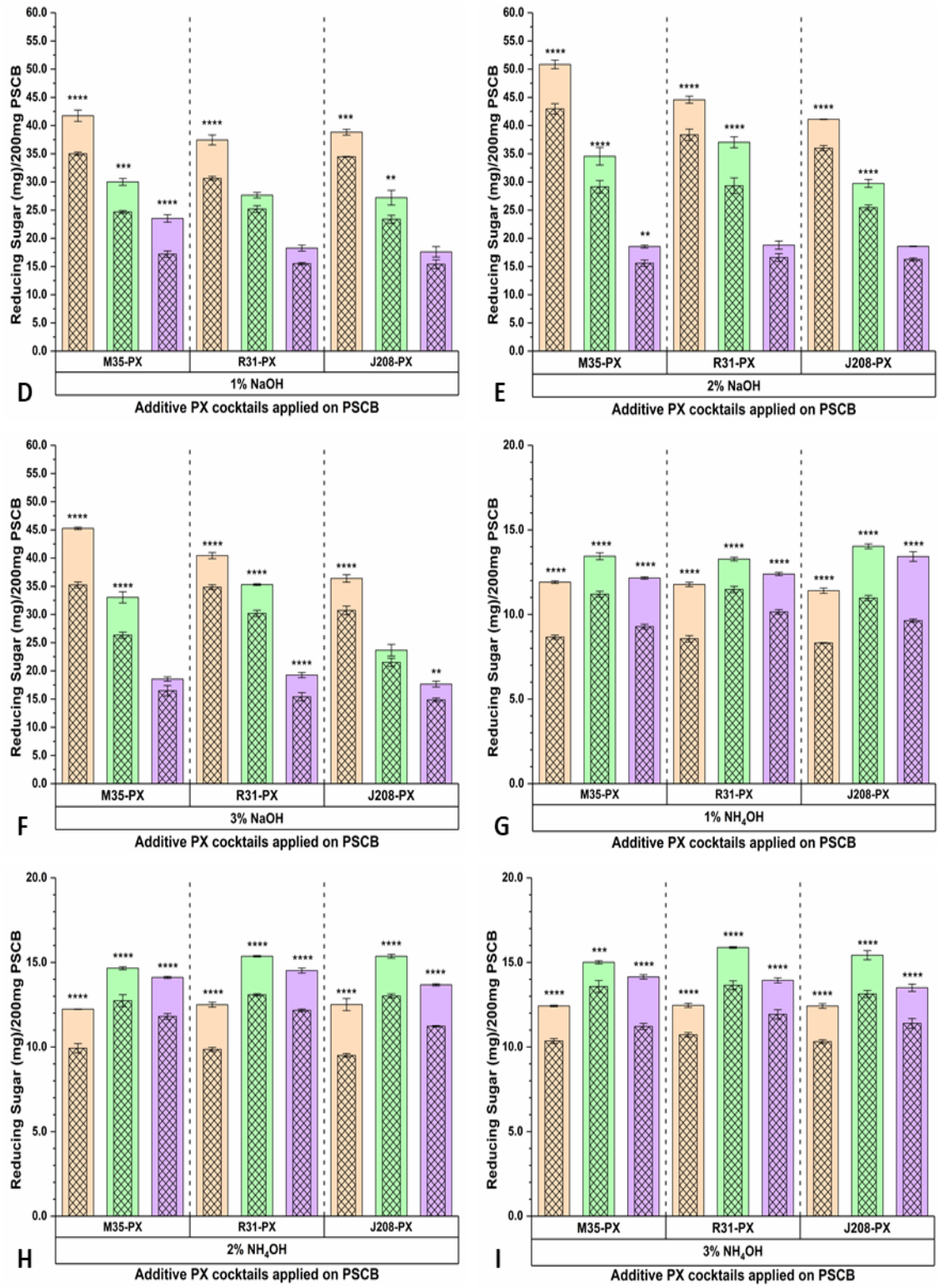
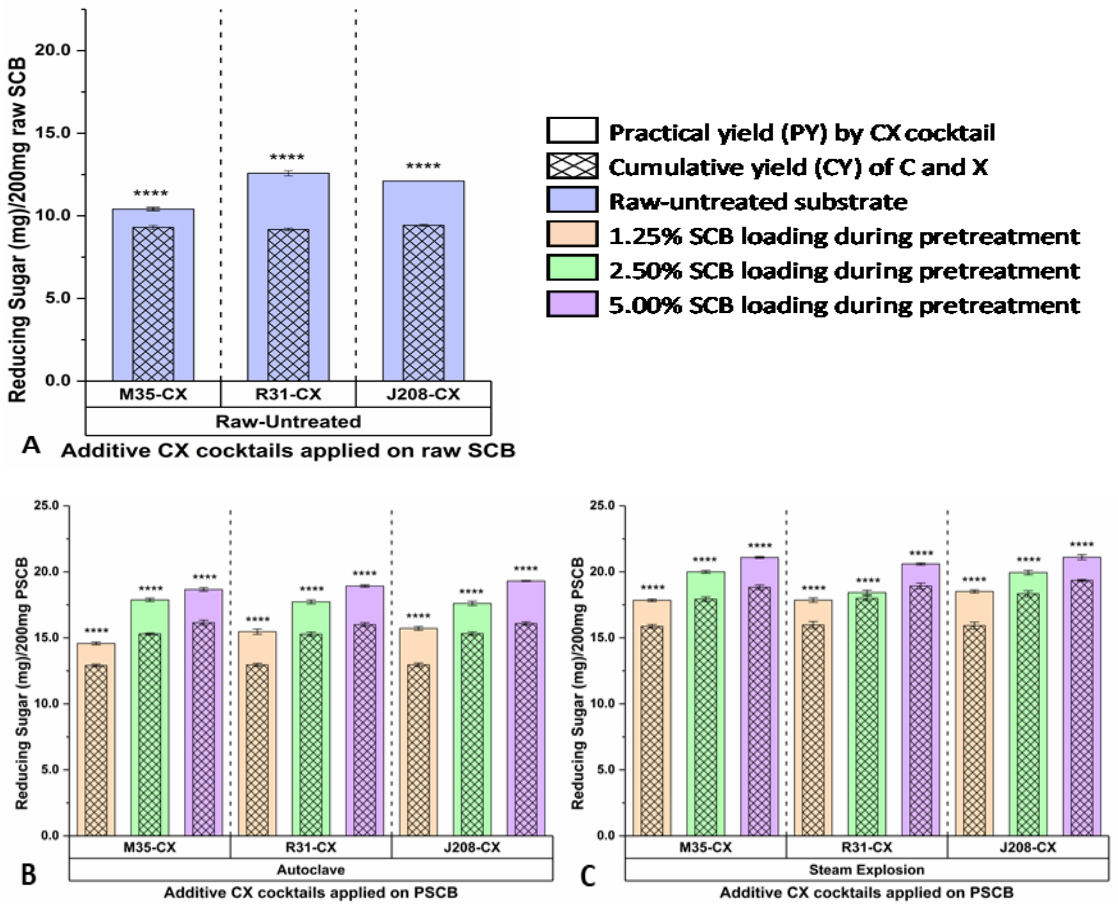


Figure 5.1. Saccharification of raw and pretreated SCB by additive PX cocktails: Synergistic effect of PX cocktail obtained by combination of pectinase (P) and xylanase (X) from *B. safensis* M35, *B. altitudinis* R31 or *B. altitudinis* J208 on hydrolysis of **(A)** Raw and **(B-H)** PSCB biomass; where, **(B)** Autoclave, **(C)** Steam Explosion, **(D)** 1% NaOH, **(E)** 2% NaOH, **(F)** 3% NaOH, **(G)** 1% NH_4OH, **(H)** 2% NH_4OH and **(I)** 3% NH_4OH; Significant difference between PY and CY is presentedd as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; Column and Error bars represents the Mean and Standard Errors of the Mean (SEM) for n=3.

5.3.1.2. Saccharification of PSCB biomass by additive CX cocktails:

As shown in Figure 5.2 (A-I), positive synergism between C and X for saccharification was observed as the practical yield by CX cocktail from each sample exceeded the cumulative yield of C and X enzymes. The difference between practical and cumulative values was significant ($p < 0.0001$) for most comparisons.

Three CX cocktails M35-CX, R31-CX and J208-CX gave practical yield in the range of ~10.4-12.7 mg from raw SCB against cumulative yield in the range of ~9.1-9.5 mg (Figure 5.2 A). Enhanced saccharification was observed in case of all pretreated substrates by each CX cocktail when compared with that of raw biomass. For autoclave and steam explosion, the practical yield by CX cocktail was observed in the range of ~14.6-21.1 mg which was higher than their cumulative yield of ~12.9-19.3 mg (Figure 5.2 B, C). NaOH pretreatment exhibited highest improvement in saccharification as the practical yield of reducing sugar after 60 h of CX cocktail treatment reached in the range of ~ 59.7-153.1 mg which was higher than their cumulative yield of ~57.7-122.2 mg (Figure 5.2 D-F). For NH_4OH pretreated biomass, the practical yield of reducing sugar after 60 h of enzymatic saccharification was calculated to be in the range of ~22.0-31.3 mg which was higher than cumulative yield of ~18.5-29.5 mg (Figure 5.2 G-I).



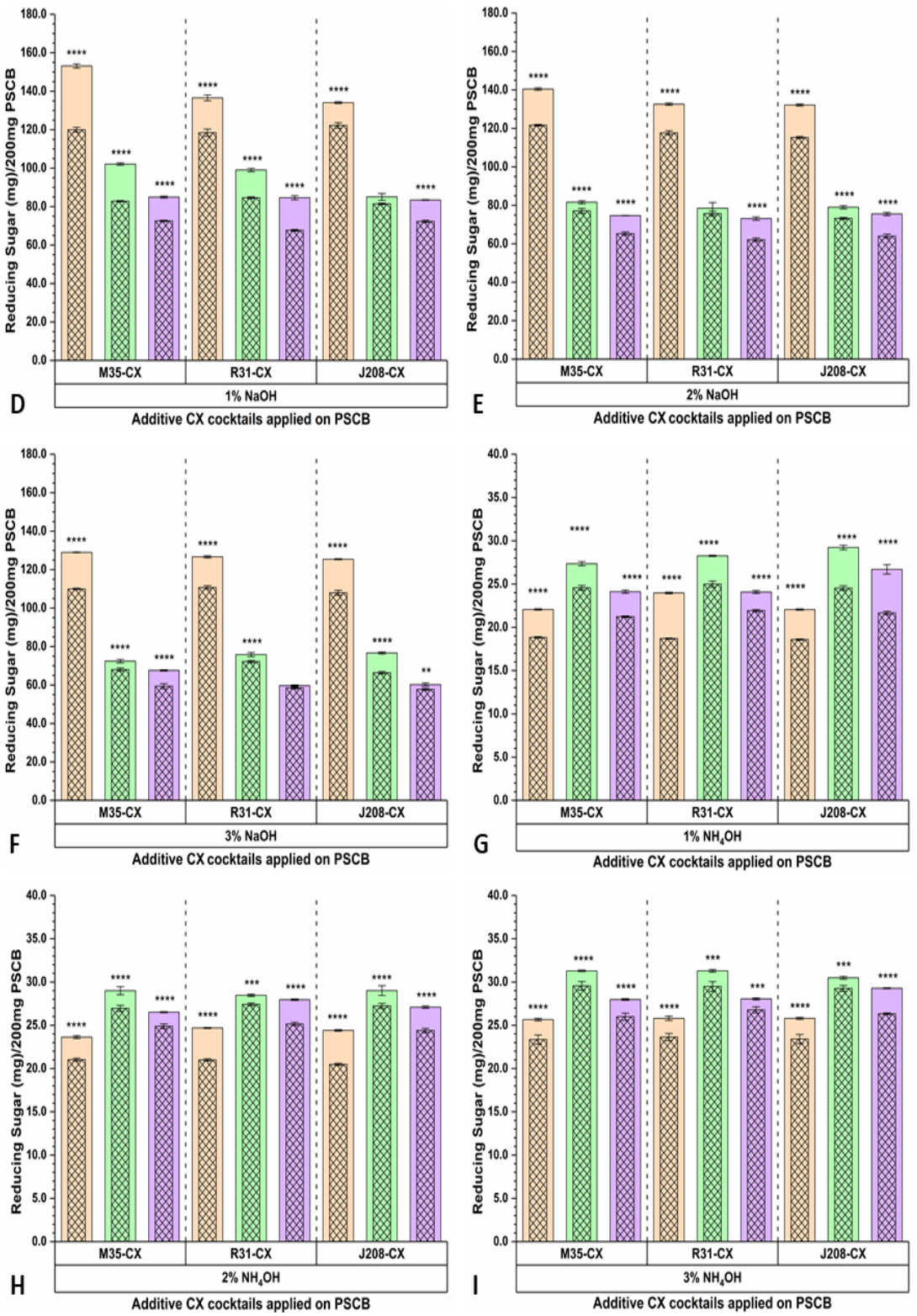
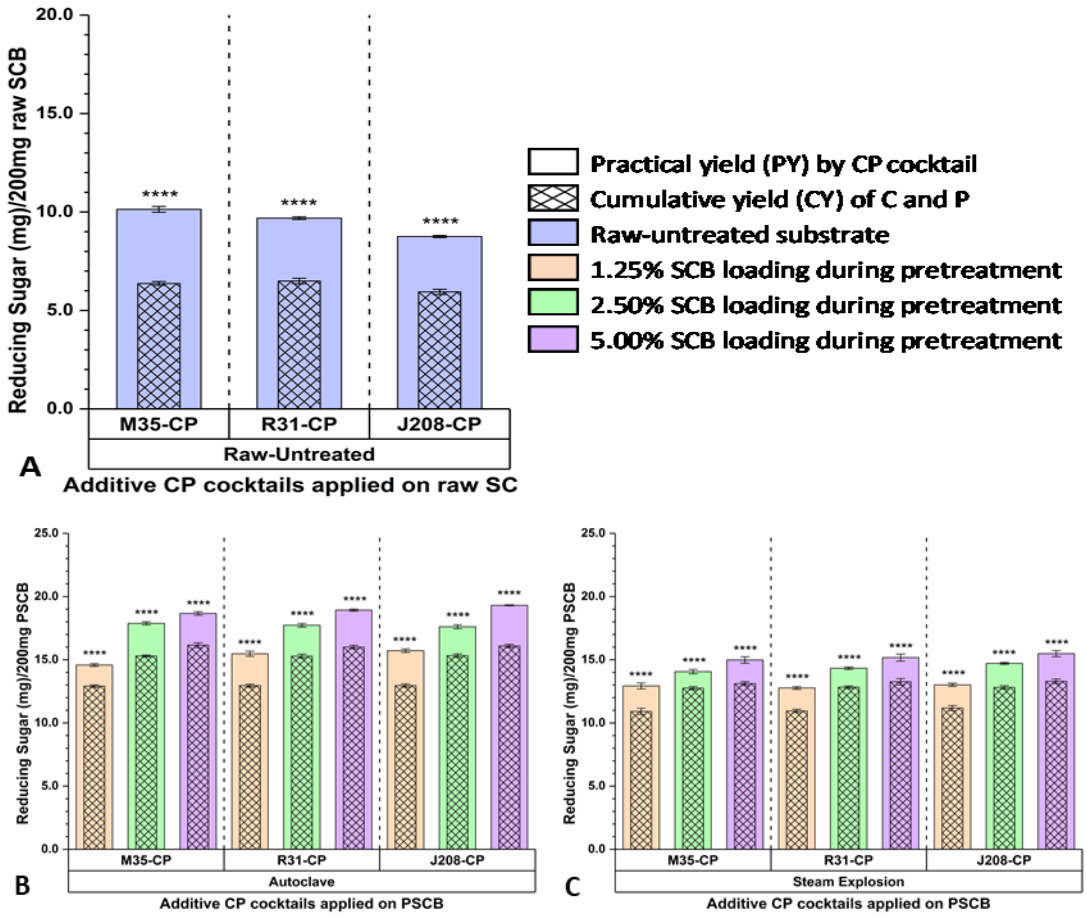


Figure 5.2. Saccharification of raw and pretreated SCB by additive CX cocktail: Synergistic effect of CX cocktail obtained by combination of commercial cellulase (C) and *B. safensis* M35, *B. altitudinis* R31 or *B. altitudinis* J208 xylanase (X), on hydrolysis of **(A)** Raw and **(B-H)** PSCB biomass; Where, **(B)** Autoclave, **(C)** Steam Explosion, **(D)** 1% NaOH, **(E)** 2% NaOH, **(F)** 3% NaOH, **(G)** 1% NH_4OH, **(H)** 2% NH_4OH and **(I)** 3% NH_4OH; Significant difference between PY and CY is presentedd as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; Columns and error bars represents the Mean and Standard Errors of the Mean (SEM) for n=3.

5.3.1.3. Saccharification of PSCB biomass by CP cocktails:

As can be seen from Figure 5.3 (A-I), positive synergism between C and P for saccharification was observed as the practical yield by CP cocktail from each sample exceeded the cumulative yield of C and P enzymes. The difference between practical and cumulative values was significant ($p < 0.0001$) for most comparisons.

Three CP cocktails M35-CP, R31-CP, J208-CP gave a practical yield in the range of ~8.7-10.2 mg from raw SCB against range of cumulative yield of ~5.9-6.5 mg (Figure 5.3 A). Saccharification by CP cocktail enhanced in case of PSCB as compared to that of raw biomass. For autoclave and steam explosion the practical yield by CP cocktail was observed in the range of ~12.9 -19.3 mg which was higher than their cumulative yield of ~10.9-16.1 mg (Figure 5.3 B, C). NaOH pretreatment exhibited highest improvement in saccharification, as the practical yield of reducing sugar after 60 h of CP cocktail treatment reached in the range of ~ 55.5-121.0 mg which was higher than their cumulative yield of ~48.0-105.1 mg (Figure 5.3 D-F). For NH_4OH pretreated biomass, the practical yield of reducing sugar after 60 h of enzymatic saccharification was calculated to be in the range of ~20.2-28.8 mg which was higher than the cumulative yield of ~16.1-23.4 mg (Figure 5.3 G-I).



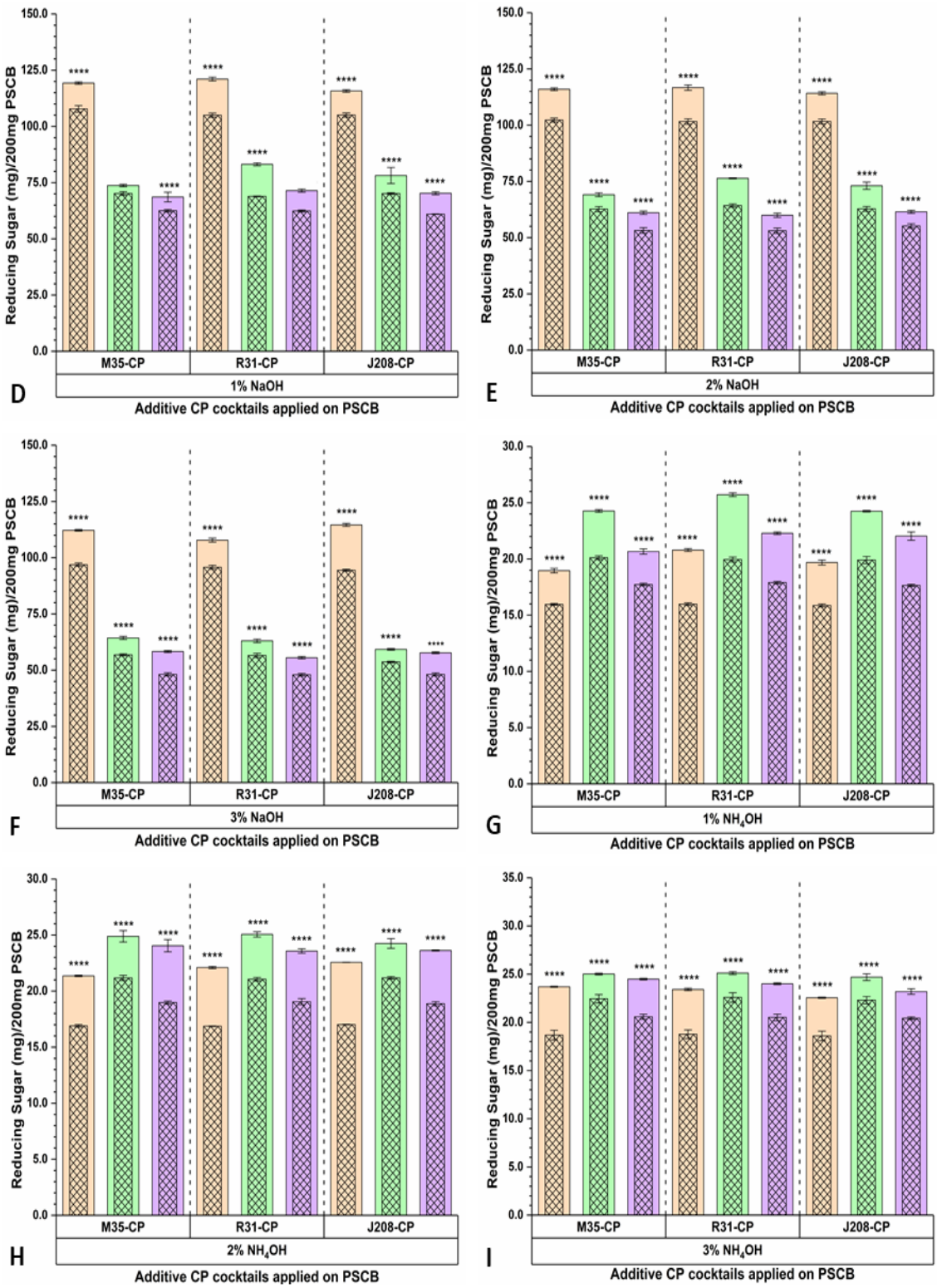
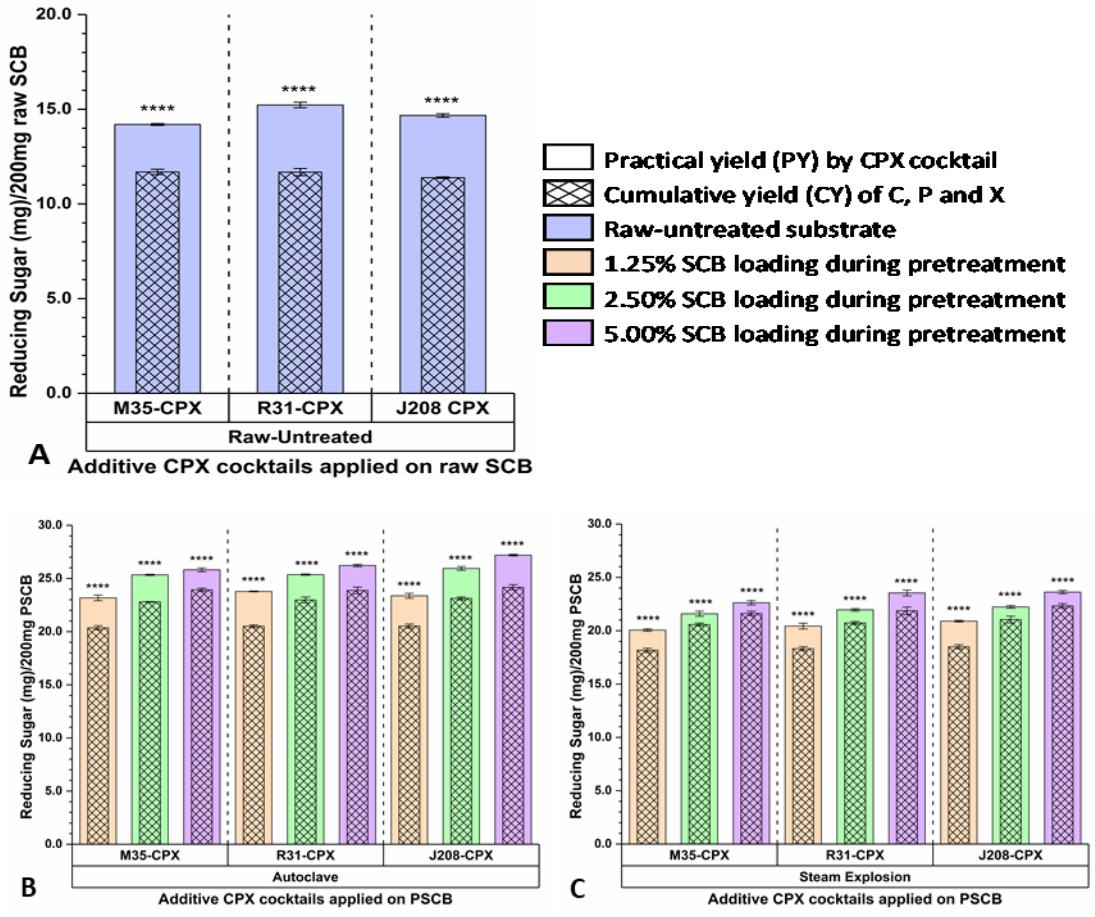


Figure 5.3. Saccharification of raw and pretreated SCB by additive CP cocktail: Synergistic effect of CX cocktail obtained by combination of commercial cellulase (C) and *B. safensis* M35, *B. altitudinis* R31 or *B. altitudinis* J208 pectinase (P), on hydrolysis of **(A)** Raw and **(B-H)** PSCB biomass; Where, **(B)** Autoclave, **(C)** Steam Explosion, **(D)** 1% NaOH, **(E)** 2% NaOH, **(F)** 3% NaOH, **(G)** 1% NH_4OH, **(H)** 2% NH_4OH and **(I)** 3% NH_4OH; Significant difference between PY and CY is presentedd as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; Columns and error bars represents the Mean and Standard Errors of the Mean (SEM) for n=3.

5.3.1.4. Saccharification of PSCB biomass by CPX cocktail:

As can be seen from Figure 5.4 (A-I), positive synergism by C, P and X for saccharification was observed as the practical yield by CPX cocktail from each sample exceeded the cumulative yield of C, P and X enzymes. The difference between practical and cumulative values was significant ($p < 0.0001$) for most comparisons.

Three CPX cocktails M35CPX, R31CPX and J208CPX gave a practical yield in the range of ~14.2-15.2 mg from raw SCB against range of cumulative yield of ~11.4-11.7 mg (Figure 5.4 A). Enhanced saccharification of each PSCB was observed for each CPX cocktail when compared with that of raw biomass. For autoclave and steam explosion the practical yield by CPX cocktail was observed in the range of ~20.0-27.2 mg which was higher than their cumulative yield of ~18.1-24.1 mg (Figure 5.4 B, C). NaOH pretreatment exhibited highest improvement in saccharification, as the practical yield of reducing sugar after 60 h of CPX cocktail treatment reached in the range of ~67.0-163.2 mg, which was higher than their cumulative yield of ~60.3-134.1 mg (Figure 5.4 D-F). For NH_4OH pretreated biomass, practical yield of reducing sugar after 60 h of enzymatic saccharification was calculated to be in the range of ~25.0-34.5 mg which was higher than the cumulative yield of ~21.3-32.7 mg (Figure 5.4 G-I).



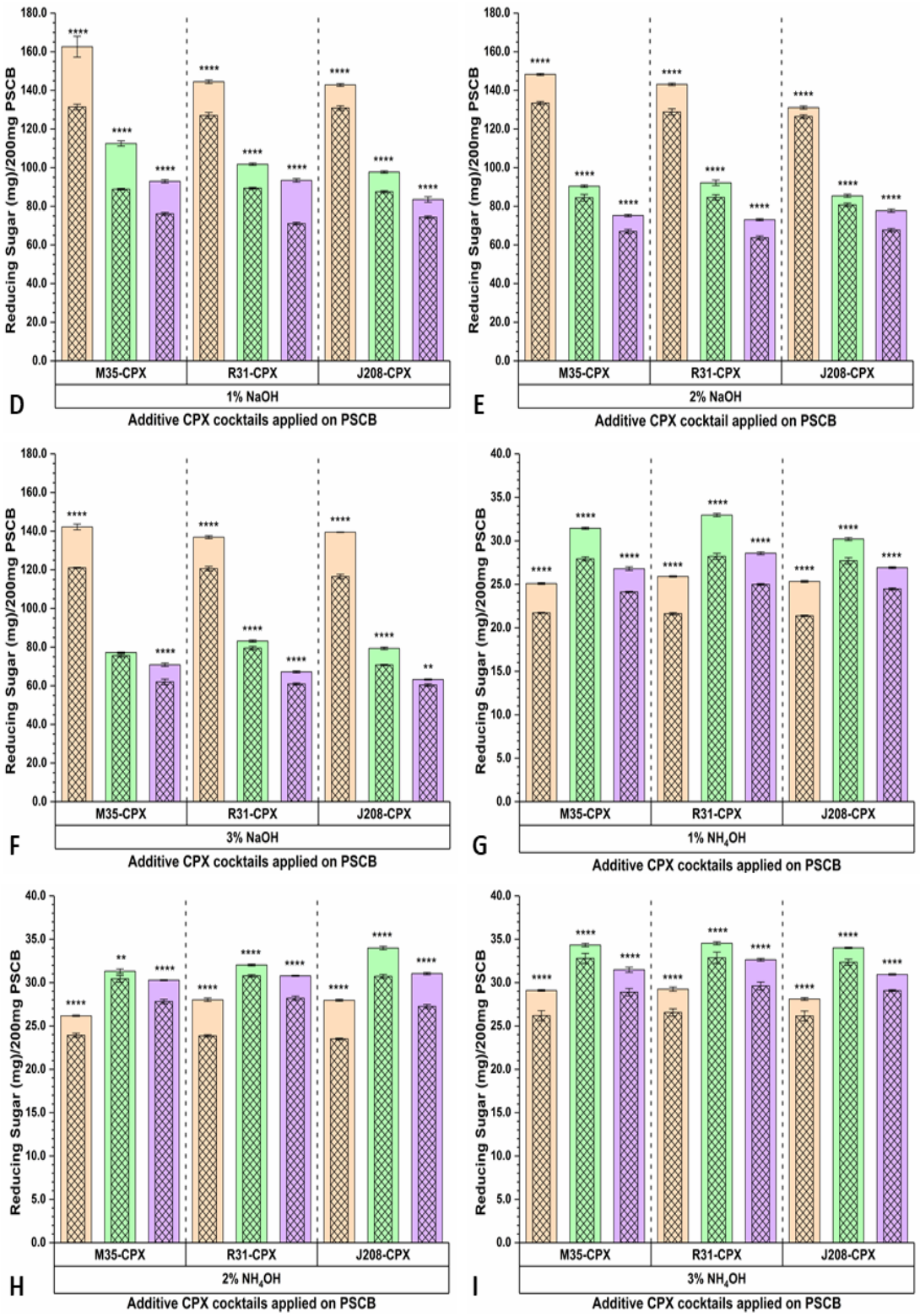


Figure 5.4. Saccharification of raw and pretreated SCB by additive CPX cocktail: Synergistic effect of CPX cocktail obtained by combination of commercial cellulase along with pectinase (P) and xylanase (X) from *B. safensis* M35, *B. altitudinis* R31 or *B. altitudinis* J208 on hydrolysis of **(A)** Raw and **(B-H)** PSCB biomass; Where, **(B)** Autoclave, **(C)** Steam Explosion, **(D)** 1% NaOH, **(E)** 2% NaOH, **(F)** 3% NaOH, **(G)** 1% NH_4OH, **(H)** 2% NH_4OH and **(I)** 3% NH_4OH; Significant difference between PY and CY is presentedd as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; Columns and error bars represents the Mean and Standard Errors of the Mean (SEM) for n=3.

5.3.1.5. Synergism in saccharification for different cocktail combinations:

Thus, after different physicochemical pretreatments, PSCB biomass exhibited enhanced saccharification by different enzyme cocktails in comparison to raw SCB. The calculated values of % saccharification and fold increase in % saccharification also known as Degree of synergism (DS) for untreated and pretreated biomass by XP, CP, CX and CPX cocktails are presented in Table 5.1, 5.2 and 5.3 respectively for M35, R31 and J208 cocktails.

As mentioned in the Section 5.1, cellulose, xylan and pectin in this order decreases in SCB biomass composition. As seen in Section 4.3.7, their accessibility to the respective enzymes was also dependent on their amount present in biomass. Thus, PX, CP, CX and CPX cocktails in this order enhanced the % saccharification of SCB. Though all PX cocktails enhanced saccharification than individual X or P enzymes, the saccharification was still less than any cellulase containing cocktail. Similarly, application of P or X or PX along with commercial cellulase C also enhanced the SCB saccharification than commercial cellulase alone.

Enzymatic treatment of raw and PSCB by each cocktail combination (PX, CX, CP and CPX) yielded a higher value of saccharification when compared with the cumulative yield of individual cocktail component enzymes i.e, (P+X), (C+X), (C+P) and (C+P+X) respectively. For all cases in present studies, the DS value > 1.0 evidently suggested effecctive synergism as the accessory and core enzymes together worked efficiently to enhance the saccharification of raw as well as pretreated biomass.

For each pretreatment, the DS values of PX, CX, CP and CPX cocktails, followed more or less a similar pattern. Where, high DS values for PX or CP cocktails suggested more positive synergism and compared to them low DS values for CX and CPX cocktails suggested less positive synergism.

Saccharification studies of finely ground raw SCB by PX, CX, CP and CPX cocktails by Li et al., (2014a) exhibited the same pattern where positive synergism obtained in the initial 2 h of enzymatic saccharification for all cocktails persisted after 48 h of incubation for CP and PX cocktails only and decreased below 1.0 for CX and CPX suggesting the lack of synergism. Similar results have been reported from NaOH, H_2SO_4 H_2O_2 treated and steam exploded SCB by same set of enzymes (Li et al., 2014b).

Table 5.1. % Saccharification and DS by various additive combinations of commercial cellulase, M35-xylanase-and M35-pectinase:

Pretreatment of SCB (%w/v)		% Saccharification and Degree of Synergism (DS) by enzyme cocktails after 60h of incubation							
		M35-PX		M35-CX		M35-CP		M35-CPX	
Method	SCB	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS
`Untreated		**4.78 ± 0.04**	**1.24**	**5.20 ± 0.07**	**1.12**	**5.07 ± 0.10**	**1.59**	**7.10 ± 0.04**	1.21
Autoclave	1.25	5.89 ± 0.07	1.12	9.63 ± 0.08	1.11	7.29 ± 0.08	1.13	11.59 ± 0.19	1.14
	2.50	6.08 ± 0.05	1.13	10.56 ± 0.06	1.08	8.94 ± 0.09	1.17	12.67 ± 0.04	1.11
	5.00	**6.34 ± 0.09**	1.11	**10.88 ± 0.28**	1.07	**9.33 ± 0.10**	1.16	**12.91 ± 0.13**	1.08
Steam Explosion	1.25	5.53 ± 0.08	1.16	8.92 ± 0.07	1.13	6.46 ± 0.16	1.18	10.03 ± 0.08	1.10
	2.50	5.94 ± 0.08	1.13	10.00 ± 0.08	1.12	7.03 ± 0.12	1.10	10.79 ± 0.17	1.05
	5.00	**6.36 ± 0.07**	1.13	**10.55 ± 0.05**	1.12	**7.49 ± 0.18**	1.14	**11.31 ± 0.14**	1.05
1% NaOH	1.25	20.87 ± 0.71	1.19	**76.56 ± 0.79**	1.28	**59.63 ± 0.37**	1.11	**81.30 ± 0.78**	1.24
	2.50	15.00 ± 0.44	1.22	51.06 ± 0.44	1.23	36.86 ± 0.37	1.05	56.25 ± 0.95	1.27
	5.00	11.77 ± 0.47	1.37	42.47 ± 0.37	1.17	34.31 ± 1.50	1.10	46.48 ± 0.58	1.22
2% NaOH	1.25	**25.42 ± 0.54**	1.18	70.23 ± 0.44	1.15	57.99 ± 0.41	1.13	74.14 ± 0.37	1.11
	2.50	17.27 ± 1.10	1.19	40.81 ± 0.56	1.06	34.54 ± 0.61	1.10	45.20 ± 0.44	1.07
	5.00	9.27 ± 0.18	1.19	37.34 ± 0.04	1.14	30.53 ± 0.51	1.15	37.61 ± 0.42	1.12
3% NaOH	1.25	22.63 ± 0.15	1.29	64.49 ± 0.11	1.17	56.09 ± 0.26	1.16	71.09 ± 1.03	1.17
	2.50	16.52 ± 0.70	1.26	36.21 ± 0.62	1.06	32.16 ± 0.47	1.13	38.59 ± 0.18	1.02
	5.00	9.27 ± 0.28	1.13	33.80 ± 0.22	1.14	29.14 ± 0.29	1.21	35.43 ±0.62	1.14
1% NH_4OH	1.25	5.96 ± 0.05	1.38	11.03 ± 0.06	1.17	9.48 ± 0.14	1.19	12.55 ± 0.07	1.16
	2.50	6.72 ± 0.14	1.20	13.68 ± 0.17	1.11	12.13 ± 0.08	1.21	15.72 ± 0.08	1.13
	5.00	6.08 ± 0.05	1.31	12.06 ± 0.13	1.14	10.33 ± 0.17	1.17	13.40 ± 0.14	1.11
2% NH_4OH	1.25	6.12 ± 0.01	1.23	11.81 ± 0.12	1.12	10.68 ± 0.04	1.26	13.09 ± 0.05	1.09
	2.50	7.33 ± 0.06	1.15	14.50 ± 0.33	1.08	12.44 ± 0.36	1.18	15.66 ± 0.18	1.03
	5.00	7.06 ± 0.04	1.20	13.26 ± 0.05	1.07	12.03 ± 0.38	1.27	15.14 ± 0.04	1.09
3% NH_4OH	1.25	6.21 ± 0.03	1.20	12.83 ± 0.10	1.10	11.85 ± 0.04	1.27	14.55 ± 0.07	1.11
	2.50	**7.50 ± 0.06**	1.11	**15.64 ± 0.06**	1.06	**12.50 ± 0.06**	1.12	**17.17 ± 0.14**	1.05
	5.00	7.07 ± 0.09	1.26	13.99 ± 0.07	1.08	12.24 ± 0.06	1.19	15.74 ± 0.21	1.09

Table 5.2. % Saccharification and DS by various additive combinations of commercial cellulase, R31-xylanase and R31-pectinase:

Pretreatment of SCB (%w/v)		% Saccharification and Degree of Synergism (DS) by enzyme cocktails after 60h of incubation							
		R31-PX		R31-CX		R31-CP		R31-CPX	
Method	SCB	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS
Untreated		**5.24 ± 0.06**	1.36	**6.29 ± 0.11**	1.37	**4.85 ± 0.05**	1.49	**7.61 ± 0.10**	1.30
Autoclave	1.25	6.09 ± 0.12	1.14	9.83 ± 0.07	1.13	7.74 ± 0.14	1.19	11.89 ± 0.03	1.16
	2.50	6.14 ± 0.04	1.12	10.71 ± 0.12	1.09	8.86 ± 0.10	1.16	12.68 ± 0.04	1.10
	5.00	**6.46 ± 0.12**	1.14	**11.07 ± 0.08**	1.08	**9.46 ± 0.06**	1.18	**13.11 ± 0.08**	1.10
Steam Explosion	1.25	5.66 ± 0.14	1.17	8.93 ± 0.12	1.12	6.38 ± 0.08	1.17	10.22 ± 0.19	1.12
	2.50	6.17 ± 0.15	1.16	9.22 ± 0.12	1.02	7.16 ± 0.07	1.12	10.98 ± 0.08	1.06
	5.00	**6.70 ± 0.12**	1.16	**10.30 ± 0.06**	1.09	**7.59 ± 0.20**	1.15	**11.77 ± 0.20**	1.08
1% NaOH	1.25	18.72 ± 0.62	1.22	**68.29 ± 1.06**	1.15	**60.52 ± 0.58**	1.15	**72.24 ± 0.64**	1.14
	2.50	13.82 ± 0.34	1.10	49.53 ± 0.62	1.17	41.58 ± 0.47	1.21	50.89 ± 0.42	1.14
	5.00	7.13 ± 0.55	1.17	42.32 ± 0.75	1.25	35.73 ± 0.47	1.15	46.72 ± 0.65	1.31
2% NaOH	1.25	**22.29 ± 0.44**	1.16	66.30 ± 0.44	1.13	58.34 ± 0.83	1.15	71.56 ± 0.46	1.11
	2.50	18.51 ± 0.69	1.27	39.25 ± 1.09	1.04	38.20 ± 0.15	1.19	46.10 ± 0.98	1.09
	5.00	6.57 ± 0.11	1.07	36.56 ± 0.62	1.18	29.97 ± 0.65	1.13	36.53 ± 0.37	1.15
3% NaOH	1.25	20.22 ± 0.39	1.16	63.32 ± 0.39	1.14	53.90 ± 0.61	1.13	68.43 ± 0.54	1.13
	2.50	17.65 ± 0.11	1.17	37.92 ± 0.72	1.05	31.51 ± 0.56	1.12	41.55 ± 0.38	1.05
	5.00	9.63 ± 0.33	1.25	29.85 ± 0.26	1.02	27.77 ± 0.37	1.16	33.59 ± 0.37	1.10
1% NH_4OH	1.25	5.89 ± 0.09	1.38	11.98 ± 0.07	1.28	10.39 ± 0.09	1.30	12.95 ± 0.05	1.20
	2.50	6.64 ± 0.08	1.16	14.13 ± 0.05	1.13	12.36 ± 0.12	1.29	16.48 ± 0.14	1.17
	5.00	6.19 ± 0.07	1.22	12.04 ± 0.12	1.10	11.14 ± 0.08	1.25	14.29 ± 0.12	1.14
2% NH_4OH	1.25	6.25 ± 0.10	1.27	12.35 ± 0.04	1.18	11.05 ± 0.07	1.31	14.01 ± 0.14	1.17
	2.50	7.68 ± 0.03	1.17	14.23 ± 0.09	1.04	12.43 ± 0.17	1.19	16.02 ± 0.07	1.04
	5.00	7.26 ± 0.11	1.19	13.98 ± 0.05	1.11	11.79 ± 0.17	1.24	15.39 ± 0.04	1.09
3% NH_4OH	1.25	6.23 ± 0.09	1.16	12.90 ± 0.17	1.09	11.71 ± 0.08	1.25	14.62 ± 0.17	1.10
	2.50	**7.94 ± 0.04**	1.16	**15.08 ± 0.30**	1.02	**12.55 ± 0.10**	1.11	**17.27 ± 0.12**	1.05
	5.00	6.97 ± 0.10	1.17	14.03 ± 0.09	1.05	12.00 ± 0.07	1.17	16.32 ± 0.11	1.10

Table 5.3. % Saccharification and DS by various additive combinations of commercial cellulase, J208-xylanase and J208-pectinase:

Pretreatment of SCB (%w/v)		% Saccharification and Degree of Synergism (DS) by enzyme cocktails after 60h of incubation							
		J208-PX		J208-CX		J208-CP		J208-CPX	
Method	SCB	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS
Untreated		**5.04 ± 0.09**	**1.36**	**6.05 ± 0.01**	**1.28**	**4.38 ± 0.04**	**1.47**	**7.34 ± 0.07**	**1.29**
Autoclave	1.25	6.36 ±0.11	1.19	10.07 ± 0.08	1.16	7.86 ± 0.10	1.21	11.69 ± 0.16	1.14
	2.50	6.46 ± 0.14	1.16	10.80 ± 0.09	1.09	8.81 ± 0.11	1.15	12.97 ± 0.12	1.12
	5.00	**6.86 ± 0.12**	**1.18**	**11.43 ± 0.06**	**1.11**	**9.65 ± 0.03**	**1.20**	**13.60 ± 0.06**	**1.13**
Steam Explosion	1.25	5.94 ± 0.19	1.20	9.26 ± 0.08	1.16	6.51 ± 0.09	1.17	10.45 ± 0.06	1.13
	2.50	6.58 ± 0.06	1.20	9.97 ± 0.12	1.09	7.36 ± 0.06	1.15	11.12 ± 0.09	1.06
	5.00	**6.91 ± 0.07**	**1.15**	**10.55 ± 0.14**	**1.09**	**7.74 ± 0.17**	**1.17**	**11.81 ± 0.11**	**1.06**
1% NaOH	1.25	19.41 ± 0.37	1.13	**67.03 ± 0.30**	1.10	**57.88 ± 0.39**	1.10	**71.42 ± 0.49**	1.09
	2.50	13.60 ± 0.92	1.17	42.53 ± 1.24	1.05	41.25 ± 0.53	1.17	48.87 ± 0.43	1.12
	5.00	8.80 ± 0.66	1.14	41.73 ± 0.13	1.15	35.14 ± 0.48	1.15	41.76 ± 0.99	1.12
2% NaOH	1.25	**20.55 ± 0.03**	1.14	66.09 ± 0.38	1.15	57.09 ± 0.49	1.12	65.54 ± 0.58	1.04
	2.50	14.87 ± 0.50	1.17	39.50 ± 0.62	1.08	36.53 ± 1.13	1.16	42.71 ± 0.58	1.06
	5.00	9.29 ± 0.03	1.14	37.75 ± 0.59	1.18	30.74 ± 0.47	1.12	38.88 ± 0.58	1.15
3% NaOH	1.25	18.19 ± 0.46	1.19	62.70 ± 0.17	1.16	57.29 ± 0.47	1.21	69.72 ± 0.11	1.20
	2.50	11.83 ± 0.73	1.11	38.34 ± 0.38	1.16	29.61 ± 0.26	1.10	39.68 ± 0.44	1.12
	5.00	8.83 ± 0.37	1.19	30.12 ± 0.59	1.04	28.87 ± 0.28	1.20	31.60 ± 0.22	1.05
1% NH_4OH	1.25	5.70 ± 0.11	1.37	11.03 ± 0.05	1.19	9.84 ± 0.15	1.24	12.66 ± 0.07	1.18
	2.50	7.02 ± 0.10	1.28	14.62 ± 0.18	1.19	12.12 ± 0.05	1.22	15.10 ± 0.12	1.09
	5.00	6.71 ± 0.20	1.39	13.35 ± 0.38	1.23	11.02 ± 0.26	1.25	13.46 ± 0.07	1.10
2% NH_4OH	1.25	6.20 ± 0.39	1.31	12.21 ± 0.07	1.19	11.28 ± 0.01	1.33	13.98 ± 0.08	1.19
	2.50	7.68 ± 0.08	1.18	14.50 ± 0.40	1.07	12.12 ± 0.30	1.14	16.99 ± 0.14	1.11
	5.00	6.84 ± 0.04	1.22	13.55 ± 0.10	1.11	11.81 ± 0.03	1.25	15.52 ± 0.10	1.14
3% NH_4OH	1.25	6.21 ± 0.10	1.20	12.90 ± 0.09	1.10	11.28 ± 0.04	1.21	14.05 ± 0.11	1.08
	2.50	**7.71 ± 0.19**	1.18	**15.24 ± 0.11**	1.04	**12.34 ± 0.24**	1.11	**17.00 ± 0.05**	1.05
	5.00	6.75 ± 0.15	1.19	14.64 ± 0.03	1.11	11.60 ± 0.20	1.14	15.47 ± 0.06	1.06

DS values for PX and CP cocktails were higher than CPX and CX cocktails, whereas highest saccharification of SCB was observed by CPX, which decreased in order of CX, CP and XP. These variations in synergism and saccharification pattern can possibly be attributed to accessibility of the polysaccharides to their enzymes which is determined by the complex interactions of structural polysaccharides. As explained earlier, the amount of cellulose and xylan are higher than pectin in the plant biomass. The pectinase probably removed pectin and enhanced the accessibility of cellulose or hemicellulose to cellulase or xylanase. So PX or CP cocktail exhibited the higher DS yet the saccharification was restricted to lower amount due to absence of cellulase in former cocktail and xylanase in later cocktail. Similarly, during simultaneous action of CX cocktail, xylanase must have enhanced the cellulose availability to cellulase by removing xylan from the matrix. And vice-versa can be said for cellulase. However, the DS values of CX were lower than PX or CP cocktails as the cellulose and xylan are present in higher amounts than pectin which resulted in higher saccharification. Similarly, it holds true for the lower DS of CPX cocktail.

The comparison amongst three combinations of hydrothermal pretreatments revealed that at 5% substrate loading both autoclave and steam explosion pretreatments

significantly enhanced saccharification. Overall the NaOH pretreatment drastically increased the saccharification than others as it removed lignin. 2% NaOH treated 1.25% SCB significantly enhanced saccharification by PX cocktail obtained from each of three isolates. While 1.25% SCB loading at 1% NaOH treatment was efficient as it significantly enhanced SCB saccharification by CP, CX and CPX cocktails and suggested this combination was the best pretreatment combination for these enzyme cocktails. Further, increased NaOH loadings or SCB loadings during pretreatment, decreased the enzymatic saccharification of SCB. Similarly, 2.5% SCB loading at 3% NH_4OH during AFEX pretreatment was efficient as it significantly enhanced SCB saccharification by PX, CP, CX and CPX cocktails obtained from the individual isolate, approving that the combination was the best for these enzymatic cocktails. Further, decreased NH_4OH concentration also decreased the saccharification.

Thus, individual M35, R31 and J208 xylanases and pectinases exhibited additive synergism amongst each other as well as with commercial cellulase when applied as PX CP, CX and CPX cocktails on PSCB, as these cocktails enhanced their saccharification. Table 5.4 represents the summery for highest saccharification by each enzyme combination from a set of each pretreatment method. As can be seen from this summary table, the highest % saccharification values obtained from NaOH PSCB, followed by NH4OH, Autoclave, steam explosion PSCB and untreated SCB for cellulase containing CP, CX and CPX cocktails. Whereas, the pattern slightly differed as NaOH followed by NH_4OH, Steam explosion, autoclave PSCB and raw SCB for accessory xylanase and pectinase containing PX cocktail. And these pretreated biomass were further opted for substitutive saccharification studies.

Table 5.4. Maximum saccharification observed for each additive cocktail from different PSCB biomass:

Enzyme cocktail		Pretreatment				
		Raw	Autoclave	SE	NaOH	NH_4OH
PX	M35	4.78 ± 0.04	6.34 ± 0.09	6.36 ± 0.07	**25.42 ± 0.54**	7.50 ± 0.06
	R31	5.24 ± 0.06	6.46 ± 0.12	6.70 ± 0.12	22.29 ± 0.44	7.94 ± 0.04
	J208	5.04 ± 0.09	6.86 ± 0.12	6.91 ± 0.07	20.55 ± 0.03	7.71 ± 0.19
CX	M35	5.20 ± 0.07	10.88 ± 0.28	10.55 ± 0.05	**76.56 ± 0.79**	15.64 ± 0.06
	R31	6.29 ± 0.11	11.07 ± 0.08	10.30 ± 0.06	68.29 ± 1.06	15.08 ± 0.30
	J208	6.05 ± 0.01	11.43 ± 0.06	10.55 ± 0.14	67.03 ± 0.30	15.24 ± 0.11
CP	M35	5.07 ± 0.10	9.33 ± 0.10	7.49 ± 0.18	59.63 ± 0.37	12.50 ± 0.06
	R31	4.85 ± 0.05	9.46 ± 0.06	7.59 ± 0.20	**60.52 ± 0.58**	12.55 ± 0.10
	J208	4.38 ± 0.04	9.65 ± 0.03	7.74 ± 0.17	57.88 ± 0.39	12.34 ± 0.24
CPX	M35	7.10 ± 0.04	12.91 ± 0.13	11.31 ± 0.14	81.30 ± 0.78	17.17 ± 0.14
	R31	7.61 ± 0.10	13.11 ± 0.08	11.77 ± 0.20	**72.24 ± 0.64**	17.27 ± 0.12
	J208	7.34 ± 0.07	13.60 ± 0.06	11.81 ± 0.11	71.42 ± 0.49	17.00 ± 0.05

5.3.2. Compatibility in substitutive cocktails of M35, R31 and J208 xylanases or pectinases:

To understand the compatibility among these three xylanases or the pectinases. comparison of % saccharification data obtained after 60 h from raw and pretreated SCB biomass by 400 µg loading of individual enzymes and substitutive cocktails were studied (Table 4.3). Application of the substituted xylanase or pectinase cocktail from M35, R31 and J208 increased % saccharification than the individual xylanases and pectinases enzymes from all raw and pretreated SCB biomass. Highest values of saccharification were observed on NaOH pretreated biomass as saccharification yield by substitutive xylanases and pectinases were 14.3 and 5.98 % when compared to saccharification values by individual enzymes. Two-Way ANOVA suggested that, though the difference between substitutive xylanase mediated saccharification and individual enzyme mediated saccharification was small, it was significant ($p < 0.001$, not shown here).

Thus, the increase in saccharification by substituted enzymes can be attributed to the compatibility of the xylanase or pectinase as well as probably due to presence of varied activities which complement each other. Therefore, the substituted cocktail of all three M35, R31 and J208 xylanases and pectinases were further studied for saccharification of raw and pretreated biomass.

Table 5.5. Comparison of % saccharification by individual xylanase and pectinase enzymes with substitutive xylanase and substitutive pectinase cocktail:

SCB biomass and pretreatment	% Saccharification			
	Individual xylanases	Substitutive xylanase	Individual pectinases	Substitutive pectinase
Raw	2.63-2.67	**2.93**	0.97-1.16	**1.32**
Autoclave	3.8-4.00	**4.21**	1.72-1.80	**2.01**
Steam Explosion	4.21-4.52	**4.74**	1.39-1.48	**1.72**
NaOH	12.6-13.6	**14.3**	5.52-5.80	**5.98**
NH_4OH	5.0-5.2	**5.47**	1.68-1.82	**1.92**

5.3.3. Substitutive saccharification studies with different cocktails of commercial cellulase and M35, R31 and J208 pectinase and M35, R31 and J208 xylanases.

After studying the additive synergism between the commercial cellulase, xylanases and pectinases the PSCB biomass was treated with enzyme cocktails formulated with substitutive approach where Figure 5.5 represents the results of reducing sugar yield by different cocktails.

As can be seen from Figure 5.5 (A-D), the saccharification of raw and pretreated biomass by PX, CX, CP and CPX cocktail enhanced when incubated up to 60 h and the difference between saccharification of pretreated and raw biomass was significant ($p < 0.001$, significance not marked in figure). The practical yield of saccharification by PX, CX, CP and CPX cocktails in case of all pretreatments exceeded the cumulative yield of individual C, P and X enzymes i.e., (P+X), (C+X), (C+P) and (C+P+X) respectively demonstrating positive synergism. The difference between practical and cumulative values were significant ($p < 0.0001$) for most comparisons.

Comparison of reducing sugar released from raw and variously pretreated SCB biomass regardless of the cocktail used in case of 1% NaOH pretreated and 1.25% SCB released highest sugars, followed by 3% NH_4OH treated and 2.5% SCB, autoclaved 5.0% SCB and steam exploded 5.0% SCB. Similarly, irrespective of the pretreatment, when reducing sugar released by different cocktails was compared, highest saccharification was exhibited by CPX cocktail followed by, CX, CP and PX. Similar results of positive synergism were obtained during the additive synergism studies presented in Section 5.3.1.

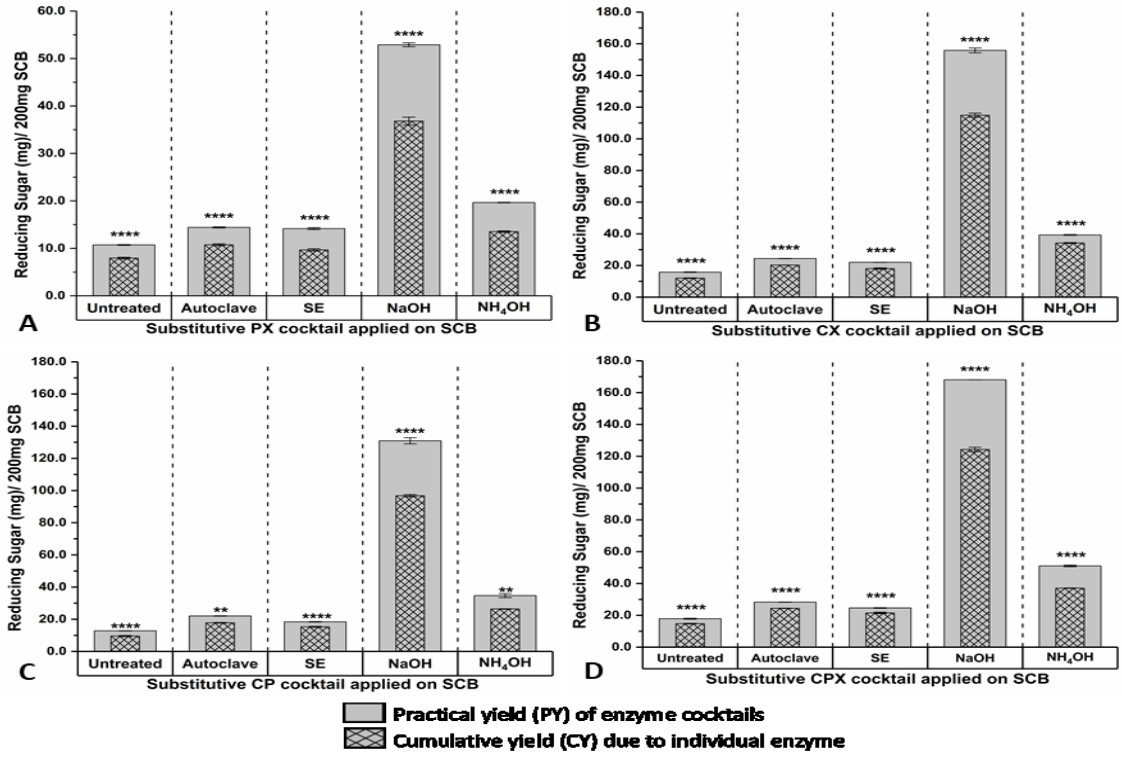


Figure 5.5. Saccharification of raw and pretreated SCB by substitutive cocktails:
Synergistic effect of substitutive ccktails **(A)** PX, pectinase-xylanase, **(B)** CX, cellulase-xylanase, **(C)** CP, cellulase-pectinase, **(D)** CPX, cellulase-pectinase-xylanase; Significant difference between PY and CY represented as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; Columns and error bars represents the Mean and Standard Errors of the Mean (SEM) n=3

Table 5.6 represents the % saccharification values of raw and PSCB by cocktails. 84.02% Saccharification of 1% NaOH treated 1.25% SCB was observed by the single CPX cocktail containing commercial cellulase and all three xylanases and three pectinases enzymes.

Table 5.6. Saccharification and DS by various substitutive combinations of commercial cellulase and xylanase-pectinase enzymes from three isolates together:

Pretreatment of SCB	% Saccharification by enzyme cocktails at 60h of incubation							
	PX		CX		CP		CPX	
	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS	% Saccharification	DS
Untreated	5.36 ± 0.06	1.35	7.90 ± 0.13	1.32	6.39 ± 0.08	1.34	8.91 ± 0.23	1.21
Autoclave	7.20 ± 0.08	1.34	12.19 ± 0.08	1.20	11.01 ± 0.10	1.24	14.15 ± 0.08	1.16
SE	7.07 ± 0.15	1.47	10.98 ± 0.04	1.22	9.19 ± 0.12	1.21	12.30 ± 0.15	1.15
NaOH	**26.44 ± 0.29**	**1.44**	**77.89 ± 1.08**	**1.36**	**65.45 ± 1.35**	**1.35**	**84.02 ± 0.04**	**1.35**
NH_4OH	9.82 ± 0.04	1.45	19.64 ± 0.20	1.15	17.33 ± 0.87	1.37	25.52 ± 0.37	1.30

Figure 5.6 represents the comparison among each of PX, CX, CP and CPX substitutive cocktail with respective additive cocktails obtained from M35, R31 and J208. The comparison suggested that the substitutive cocktail enhanced saccharification compared to respective additive cocktails in all four cases. As can be seen from Figure 5.6 (A-D), the difference between saccharification by substitutive cocktail and each additive cocktail was significant for NaOH as well as NH_4OH pretreatments irrespective of the involvement of C, P and/or X in cocktails. The pattern of saccharification by substitutive cocktail followed the same order i.e., CPX, CX, CP, PX in which the saccharification decreased similar to the pattern observed for additive cocktails. For CP combination of cocktails (Figure 5.6 C), substitutive cocktail enhanced the saccharification of each pretreated biomass significantly when compared with individual additive cocktail. As expected the highest saccharification was exhibited by CPX cocktails (Figure 5.6 D). It can be seen that substituted CPX cocktail exhibited 84.02 % saccharification against 81.30, 71.42, 72.24 % saccharification obtained by M35-CPX, R31-CPX and J208-CPX additive cocktails respectively for NaOH PSCB which was highest in studies presented here. Whereas, for NH_4OH PSCB, substituted CPX cocktail yielded saccharification up to 25.52%, against 17.17, 17.27 and 17.00 % saccharification obtained by M35-CPX, R31-CPX and J208-CPX cocktails respectively (Figure 5.6 D).

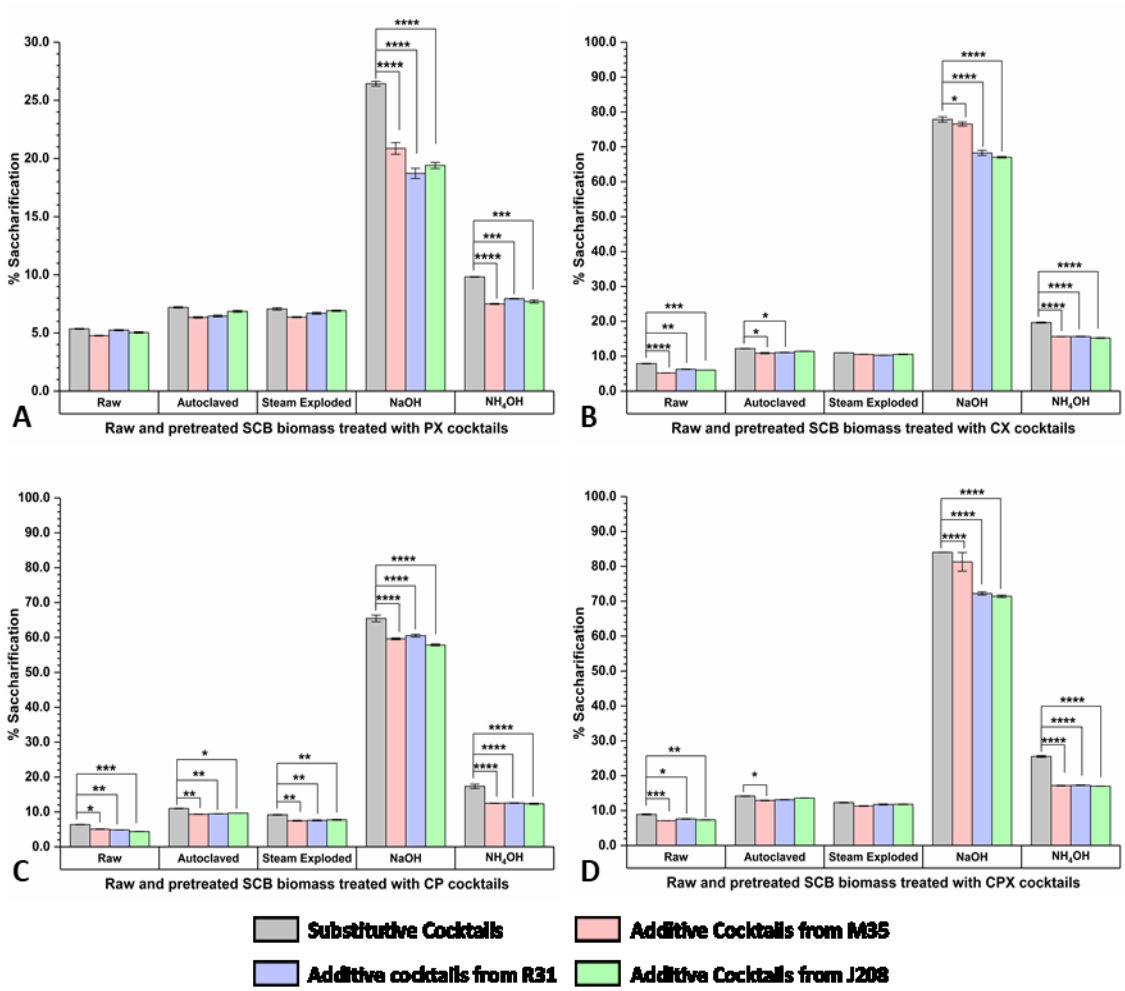


Figure 5.6. Comparison between PSCB saccharification by substitutive and additive cocktails:
(A-D): Comparison of saccharification obtained by **(A)** PX, **(B)** CX, **(C)** CP and **(D)** CPX cocktails; Error bars represented as Standard Errors of the Mean (SEM); Significant difference between additive and substitutive cocktail is given as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; for n=3.

Thus, above results suggested that, the substitutive cocktail can more efficiently enhance the saccharification of biomass than additive cocktail. Hence cellulase and substituted CPX treated SCB biomass was further selected for structural studies.

5.3.4. Structural analysis of PSCB biomass before and after treatment with commercial cellulase and substituted CPX cocktail:

The changes in chemical composition and structure of cellulase and substituted CPX cocktail treated PSCB biomass was analyzed using FTIR and SEM techniques.

5.3.4.1. FTIR analysis of enzyme hydrolysed PSCB biomass samples:

When the FTIR spectra of before and after treatment with cellulase (C) alone and CPX cocktail were compared for individual pretreated samples, as can be seen in Figure 5.7, changes in the several peaks were observed. As explained in Table 3.6, the

absorbance peaks observed at 2950-2820 cm^{-1} are attributed to -C-H stretching which can be observed as symmetrical and asymmetrical stretching vibration for $-CH_3$, $-CH_2$ or -CH group containing compounds and can be correlated with the individual monomer of polysaccharides, where -C-H bond can be considered as a specific characteristic of aldehyde carbon (Yang et al., 2007). Decrease in intensity of this peak after CPX cocktail treatment was more than it was in case of only cellulase treatment as expected. In all pretreatments, the CPX cocktail have saccharified the biomass substantially as decrease in 2950-2820 cm^{-1} peak was prominent. This can be corelated to decreased amount of carbohydrate components due to enhanced biomass saccharification. This also correlated with the earlier observation of saccharification studies, where CPX gave more saccharification as compared to Cellulase. The intensities of these peaks were unaffected in case of raw SCB biomass (Section 3.3.4.2 of Chapter 3) and PSCB biomass (Section 4.3.6.1 of Chapter 4) without any enzymatic saccharification.

The area of 1800 cm^{-1} to 800 cm^{-1} in FTIR spectra 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). The peaks at wavenumbers 1734, 1600, 1376, 1320, 1270, 1160 and 1120 were observed to change before and after the enzymatic treatment for each of the pretreated biomass. The peaks at 1734, 1600 and 1270 cm^{-1} were present for autoclave and steam explosion PSCB but were absent from NaOH and NH_4OH PSCB. The decrease in their intensity after application of commercial cellulase was further enhanced when cellulase was applied as a CPX cocktail. Further analysis suggested that, application of crude xylanase might have broken down the linkages between xylan and lignin, and freed lignin and providing the space to cellulase. Whereas, 1376, 1320, 1160 and 1120 are the peaks specifically attributed to the features of hemicellulose and cellulose polysaccharides. As can be seen from Figure 5.7 for all four pretreatments, the decrease in above mentioned peaks was more after CPX cocktail treatment when compared to commercial cellulase treatment alone.

Thus, observations of FTIR analysis supported the earlier results of presented studies suggesting that the application of all three xylanases as well as pectinases along with commercial cellulase enhanced the biomass saccharification.

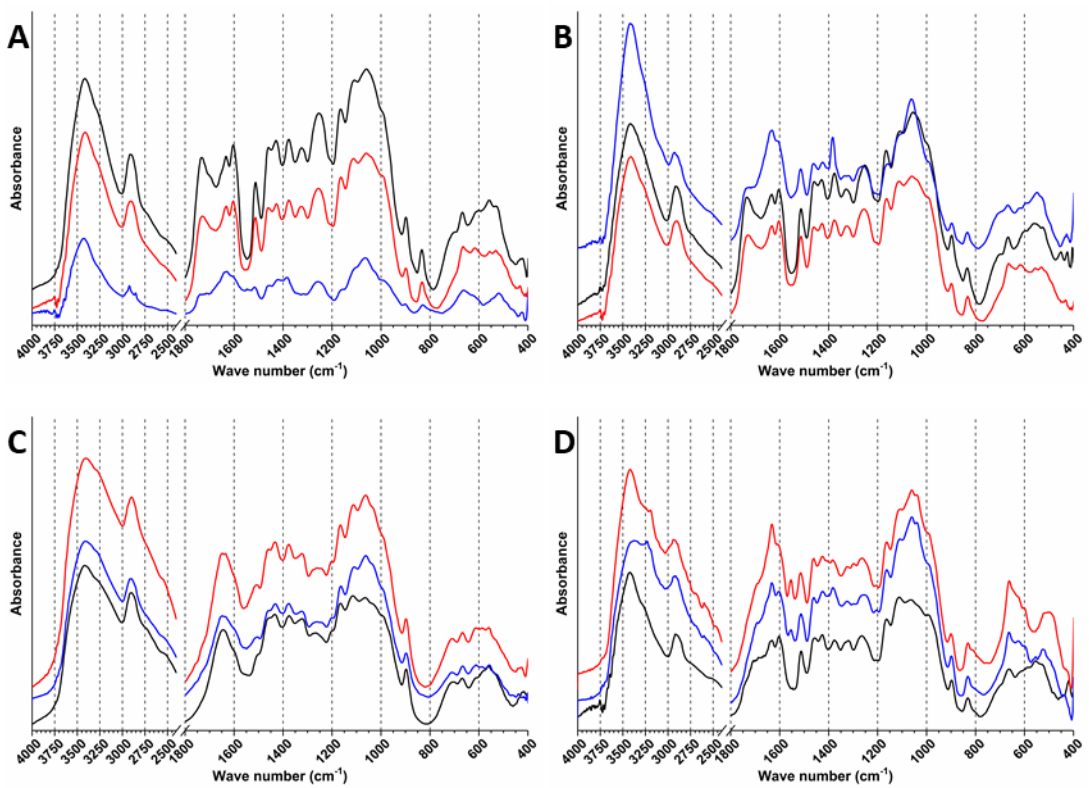


Figure 5.7. FTIR spectra of PSCB biomass solids before and after enzymatic saccharification:
(A) Autoclave PSCB, **(B)** Steam explosion PSCB, **(C)** NaOH PSCB and **(D)** NH_4OH PSCB before any enzymatic treatment (black, ▬), after treatment up to 60 h with commercial cellulase (red, ▬) and substituted CPX cocktail (blue, ▬); Y-axis is presented as an offset.

5.3.4.2. SEM analysis of enzyme hydrolysed PSCB biomass samples:

As can be seen for SCB biomass treated with the autoclave (Figure 5.8 A, B), steam explosion (Figure 5.8 C, D) and NH_4OH (Figure 5.8 G, H), the substituted CPX treated biomass exhibited more shredded and scaly appearance (Figure 5.8, B, D & H) than only cellulase treated biomass (Figure 5.8 A, C & G). The parenchymatous cells of the soft pith region from the biomass were majorly affected. More flaky appearance of these cells was clearly visible in case of steam explosion followed by NH_4OH and autoclaved. This appearance is probably a function of efficient breakdown of cell walls due to synergistic actions of cellulase, xylanase and pectinase enzymes, as the accessibility of cellulase is restricted due to presence of complex cellulosic-hemicellulosic polysaccharide matrix. Whereas in case of NaOH pretreated biomass, the roughness of shattered fibre and flaky appearance in some regions were observed which enhanced with CPX treatment than cellulase alone. As explained in previous sections, several scientists have reported such changes in morphologies of different biomass after pretreatments (C. Rezende et al., 2011; Kuila and Banerjee, 2014; Rajak and Banerjee, 2016).

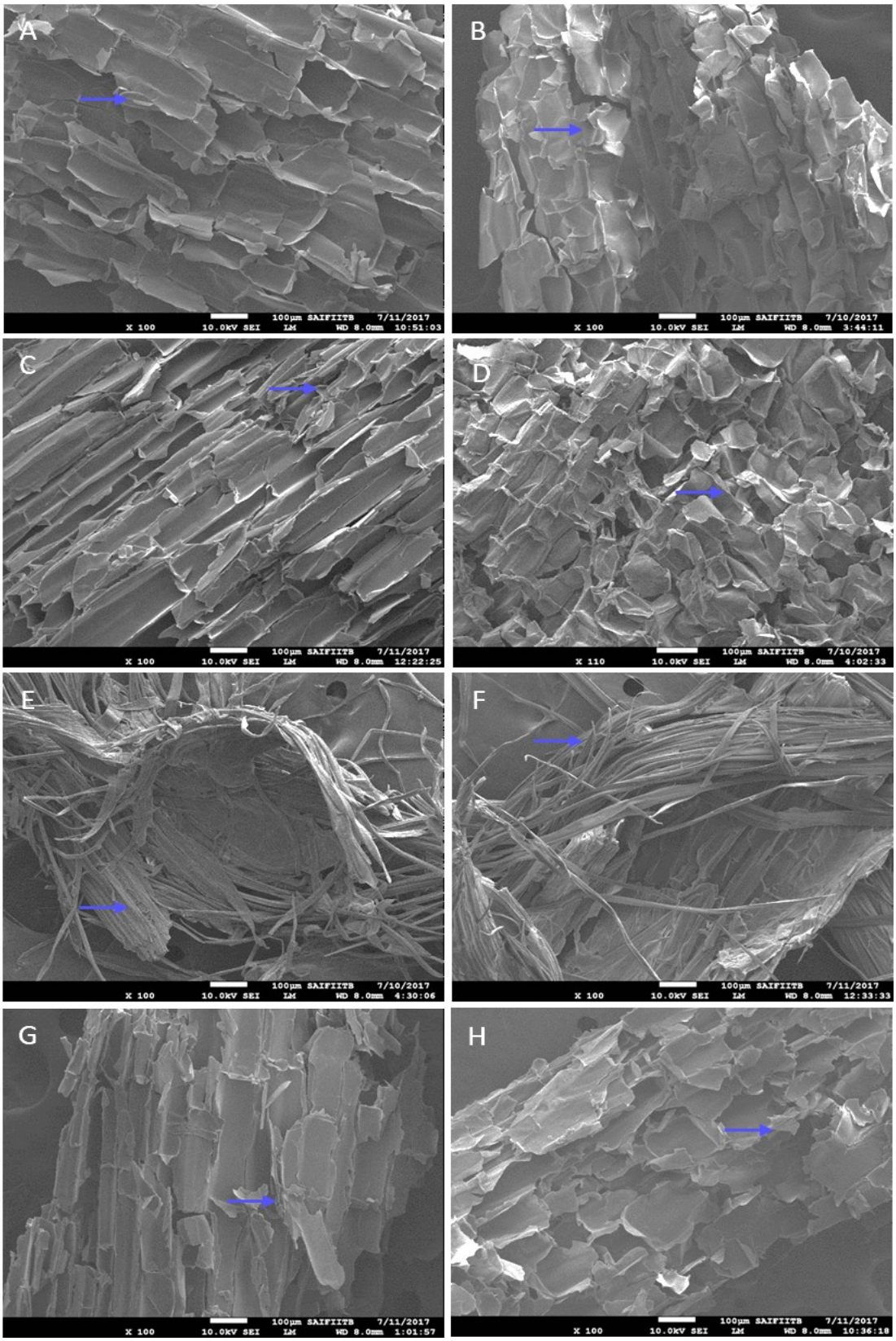


Figure 5.8. Scanning electron micrographs of PSCB after enzymatic hydrolysis with cellulase and substituted CPX cocktail:
(A,B) Autoclaved; **(C,D)** Steam exploded; **(E,F)** NaOH treated; **(G,H)** NH_4OH treated SCB biomass fragments;**)**: Images taken after 60 h of saccharification by commercial cellulase **(A, C, E** & **F)**; Images taken after 60 h of saccharification by substituted CPX cocktail **(B,D, F** & **H)**; All images were captured at 100X magnification (white bar represents scale of 100µm).

FTIR and SEM analysis confirmed that the substitution of xylanase and pectinase in commercial cellulase containing cocktails improves the saccharification of biomass and this improvement is more significant after pretreatments of SCB biomass.

Number of reports regarding applications of commercial cellulases along with commercial xylanase and pectinases are available from different sources for saccharification of varied agrowaste biomass. In most of the studies the work has been carried out using commercial cellulase containing Celluclast 1.5 L, Novozyme and188 Multifect xylanase and pectinase enzymes as mentioned in Chapter 1. A few reports are also available of crude enzymes from isolated microorganisms while in some cases commercial core cellulase is supplemented with crude enzymes from isolates. Table 5.7 presents a comparative data for agrowaste saccharification from such reported cocktails and those from present work.

Table 5.7. Saccharification of pretreated agrowaste biomass by diverse enzyme cocktails:

Organisms enzyme	Enzymes	Substrate and	Saccahrification	References
Commercial cellulase	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-xylanase	H_2O_2 pretreated SCB	~23.8%	Li et al., (2014b)
Commercial cellulase	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-xylanase	H_2SO_4 pretreated SCB	~30.7%	Li et al., (2014b)
Commercial cellulase	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-xylanase	Steam exploded SCB	~77.7%	Li et al., (2014b)
Commercial enzymes	Celluclast 1.5 L and Novozyme 188	Chlorite pretreated *L. camara*, *P. juliflora*, and Corn cob	86.4-91%	Gupta et al., (2011)
Commercial cellulase	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-xylanase	Alkali treated SCB	~79.7%	Li et al., (2014b)
Commercial enzymes	Spezyme CP, Multifect Xylanase, Multifect Pectinase	AFEX pretreated	~80-85%	Garlock et al., (2012)
Bacillus pumilus strain MK001	Xylanase with commercial cellulase and cellobiase	Alkali pretreated *P. juliflora*	~20%	Kapoor et al., (2001)
Chrysoporthe cubensis	Crude enzyme containing Cellulase, endoglucanase, xylanse, pectinase, laccase etc..,	Alkali pretreated sugarcane bagasse	~57%	Maitan-Alfenas et al., (2015a)
B. licheniformis	Accellrase1500 and Galactanase (Bl1609Gal)	Delignified SCB	~25%	Antonio De Lima et al., (2010)
B. safensis M35, *B. altitudinis* R31 and *B. altitudinis* J208	Substitutive cocktail of Commercial Primafast®200, Xylanase, Pectinase	NaOH pretreated SCB	84.03%	This study

In case of chlorite pretreated biomass, highest value of 84-91% saccharification was observed by commercial cellulase cocktails. Assistance of Mulifect pectinase and Multifect xylanase to the commercial cellulase Spezyme CP enhanced saccharification

up to 80-85% in optmimized condition. Thes saccharification values were comparable with the saccharification of 84.03% achieved from 1% NaOH PSCB by substitutive cocktail of M35, R31 and J208 xylanases and pectinases and commercial cellulase Primfast®200 in present studies. Commercial cellulase, Celluclast and Novozyme 188 have been reported to require the assistance of accessory enzymes (Zhao et al., 2009; Krishnan et al., 2010; Silva et al., 2017).In the studies cited in table 5.6 the commercial cocktails achieved appreciable saccharification with highest value being 91%. However, among the isolates the substitutive cocktails used in present study gave highest 84.02% saccharification.

Thus, the accessory role of individual xylanase and pectinase enzymes from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 was confirmed as they improved the biomass saccharification from ~7% to ~84% by enhancing the accessibility of cell wall polysaccharides components of pretreated biomass to their respective saccharifying enzymes. These studies definitely exhibit a potential of crude xylanases and pectinase from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 to work as accessory enzymes and provide a scope for formulations of diverse enzymatic cocktails involving these enzymes, along with other core or accessory enzymes for use in biomass saccharification industries.