Chapter 3

Synergism of crude xylanases and/or pectinases with commercial cellulase in enzyme cocktail mediated saccharification of raw agrowaste biomass

3.1. Introduction:

B. safensis M35, *B. altitudinis* R31 and *B. altitudinis* J208 isolated respectively from camel, cow and buffalo dung samples were selected for further studies. Reports mention that *B. safensis* and *B. altitudinis* have been isolated from a wide range of habitats such as Himalayan higher altitudes, space craft assembly facility, rhizosphere of diverse plants, whey of meat and milk, desert, organic compost, ruminant dungs, decaying wood, hydrocarbon contaminated soil, oil palm meal, marine sediments, pond water, fermented seeds and food, activated sludge, heavy metal mines, tannery effluent, insect gut, feather dump site and many others (Satomi et al., 2006; Shivaji et al., 2006; Chi et al., 2012; Adhyaru et al., 2014; Lateef et al., 2015). Survival in such diverse habitats is due to the unique physiological and genotypic characteristics of these bacteria. Lateef et al., (2015) have summarised various biotechnological applications such as plant growth-promoting bacterium, bio-control agents against plant pathogens, probiotic organism and bioremediating organism as well as capabilities of producing several industrial enzymes highlighting the importance of *B. safensis*. Although several reports are available on production and application of industry related enzymes from *B. altitudinis,* a review similar to *B. safensis* is lacking. Both *B. safensis* and *B. altitudinis* catagorized under biosafety level-1, exhibit several industrially important traits such as production of variety of enzymes like protease, lipase, polysaccharide hydrolases, ligninases; several biochemicals like bio-surfactants, several traits like plant growth promoting rhizobacteria (PGPR), bio-control, probiotics and bioremediation, etc, (Kumar et al., 2011; Vijay Kumar et al., 2011; Madhuri et al., 2012; Mao et al., 2013; Lateef et al., 2015; Sunar et al., 2015).

Reports regarding diverse applications of xylanase and pectinase from varied microbial sources are plenty. Food processing, treatments of waste waters from juice and paper industries, textile processing, degumming of plant bast fibres, coffee and tea fermentations, plant protoplast isolation, plant biomass breakdown for biofuels and many others fields have been advanced with applications of xylanase and/or pectinase enzymes (Beg et al., 2001; Hoondal et al., 2002; Aoyagi, 2011; Rebello et al., 2017; Walia et al., 2017). Production of organic acids, protein-enriched feeds, ethanol, several aroma compounds, and natural antioxidants etc., from the agro-industrial waste can be improved by the enzymatic pretreatment of the raw material by one or several microbial enzymes such as cellulase, pectinases, pectin methyl esterase, xylanases, acetyl-xylan

esterases, phenolic acid esterases, and lignocellulolytic enzymes etc, (Gassara et al., 2010).

Agrowaste from different plants possess variations in their composition of cellulose, hemicellulose, pectin, lignin. As mentioned in Section 1.4, Chapter 1, the covalent and noncovalent interactions among these macromolecules also create diversities in complex matrix of plant cell wall structural polysaccharides (Krawczyk et al., 2008; Bledzki et al., 2010). Enzymatic hydrolysis of such agrowaste plant residues by "exo- and endo-cellulases of core enzyme group" is the first saccharification step in the process of ethanol production which requires assistance of some "accessory or auxiliary group of enzymes" such as xylanases and pectinases to obtain best possible saccharification (Hu et al., 2011; Delabona et al., 2013; Li et al., 2014b). Simply stated the accessory enzymes like xylanases and pectinases unmask the cellulose from matrix in the plant biomass by hydrolysing the hemicellulosic and pectic shield for the action of the core cellulolytic enzymes to enhance the sugar release. Maitan-Alfenas et al., (2015) have reported improved saccharification of wheat bran and sugarcane bagasse by the enzyme cocktail containing cellulase, pectinase and xylanase, all three produced by fungus *Chrysoporthe cubensis*.

Cellulases which carryout saccharification of major plant polysaccharide component from plant cell wall usually drive the process of saccharification and are called "core" enzymes. The "core" group consists of several enzymes like, endoglucanase, β-1,4-glucosidase, cellobiohydrolase, etc., which can lead the saccharification process yielding glucose. To enhance their activity by availing more amount of cellulose from complex matrix, the "accessory" group of enzymes are required. These accessory enzymes free the cellulose fibres from hemicellulose covering by removing it and enhance cellulose accessibility and digestibility by core cellulase (Banerjee et al., 2010). Thus, to enhance the saccharification process, core and accessory group of enzymes are applied together. This mixture of different enzymes is commonly known as enzyme cocktail or enzyme concoction.

An outcome of core and accessory enzymes containing cocktail application during agrowaste saccharification depends on the synergism between two different enzymes. In a simplified way, synergism means when two or more enzymes are applied together, they work better than either of the individual enzyme. In this context, when enzyme cocktail is applied, the component enzymes acts on different sites of one or more substrates and thus, their combined action is enhanced as action of each one

creates new sites to attack for the other enzymes in the cocktail. Thus, the synergism can be studied for three different combinations, i.e., (i) Synergism between "core" cellulase groups of enzymes, (ii) Synergism between "core" and "accessory" groups of enzymes and (iii) Synergism between different "accessory" groups of enzymes.

Based on the above information, the study was aimed to understand the synergism of these crude xylanases and pectinases obtained from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 with core commercial cellulase for biomass saccharification. Enzyme cocktail mediated saccharification of agrowaste biomass was performed with suitable cocktails formulated through supplementation of crude xylanase and/or pectinase enzymes and/or core commercial cellulase.

3.2. Materials and Methods:

3.2.1. Chemicals, crude polysaccharide substrates, enzymes and their producers:

All required chemicals, purchased from HiMedia (Mumbai, India) or Sigma-Aldrich (Missouri, USA) or SRL Pvt. Ltd. (Mumbai, India) and were of assay grade. Citrus Peel (CP) and Wheat Bran (WB) were collected from local market, processed and stored as mentioned in Chapter 2, Section 2.2.1. WB and CP were used as crude polysaccharide substrates in media for selective production of xylanase and pectinase enzymes respectively.

Three bacterial cultures, *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *Bacillus altitudinis* J208 were used as source of xylanse and pectinase enzymes for the assessment of their synergistic role in this chapter. Isolates were maintained by inoculation and incubation on Nutrient Agar (NA) plates at 37 °C for 24 h followed by storage at 4-6 °C. Xylanases and pectinases, produced by these three isolates on the production media containing WB and CP respectively were used as crude enzymes and will be stated with the prefix of the bacterial strain they are obtained from. Thus, crude xylanases are referred to as M35 xylanase, R31 xylanase, J208 xylanase and crude pectinases are referred to as M35 pectinase, R31 pectinase and J208 pectinase throughout the studies. Primafast®200 (Genencore, Du-Pont) cellulase was suitably diluted and used as crude source of cellulase for saccharification studies and is referred as commercial cellulase throughout the studies.

Three raw agrowaste biomass materials used for saccharification studies were procured from local places near Vadodara, and included Barley Husk (BH), Sugarcane

Bagasse (SCB) and Wheat Husk (WH). SCB is an industrial waste biomass product left over after squeezing the juice out of sugarcane for sugar processing, while BH and WH are agricultural waste products left over after threshing the fully-grown grains from their covering from the harvested cobs.

3.2.2. Enzyme production for cocktail mediated biomass saccharification:

The biomass saccharification studies were performed using cell free supernatant (CFS) as a crude source of xylanase and pectinase obtained from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 grown on WB and CP respectively. Hence, usage of terms xylanase and pectinase ahead throughout the studies denote crude enzyme.

3.2.2.1. Media for enzyme production under submerged fermentation:

B. safensis M35, *B. altitudinis* R31 and *B. altitudinis* J208 cultures were separately inoculated into 10 ml of nutrient broth and incubated at 37 °C at 160 rpm till their optical density (OD_{600nm}) reached to 0.2. 0.5% of this was inoculated into 150 ml of BHM-YEP medium containing (g/L) of Bushnell Haas Medium (BHM): 3.27, Yeast extract (YE): 0.25, Peptone (P): 0.75 and crude substrate either WB or CP (as 0.5% w/v) in 500 ml Erlenmeyer flask, incubated at 37 °C for 36 h in shaking condition at 160 rpm for production of xylanase or pectinase respectively. Media was sterilized by autoclaving at 10 lbs for 20 min. Grown cultures were then centrifuged at 10,000 rpm for 20 min, and cell free supernatant (CFS) was further used as crude enzymes source of xylanase or pectinase for synergism studies.

3.2.2.2. Protein estimation by Bradford method:

CFS was diluted appropriately using Distilled Water (DW) to 1.0 ml and to this 1.0 ml of Bradford reagent was added. After incubation in dark for 10 min, absorbance of the reaction mixture was measured at 595 nm. Bovine serum albumin was used as standard based on which the amount of protein equivalent to BSA was calculated (adapted and modified from Bradford, 1976; Kruger, 2002).

3.2.3. Agrowaste biomass saccharification by enzyme cocktails:

Barley Husk (BH), Sugarcane Bagasse (SCB) and Wheat Husk (WH) were washed, dried, partially ground and stored in separate airtight containers. 2.0% w/v of individual agrowaste biomass substrate was suspended in 10 ml of 50 mM Tris-Cl buffer pH 7.0 for cocktail mediated enzymatic saccharification studies. 100 µg/ml each of Sodium Azide, Ampicillin, Kanamycin and Streptomycin was amended in this to

prevent microbial contamination. Biomass saccharification assays with individual enzymes (C, P and X), combination of two enzymes (CP, CX and PX), and all three enzymes together (CPX) were performed separately for each agro-waste BH, SCB and WH. 0.32 mg (in terms of protein) loading of commercial core cellulase per 200 mg of dry agrowaste biomass was maintained constant throughout the studies. Two separate experiments were performed with loadings of either 0.2 mg or 0.4 mg (in terms of protein) xylanase and/or pectinase from individual *Bacillus* isolates termed as P_2 or P_4 and X_2 or X_4 respectively per 200 mg of dry agrowaste biomass. Each experimental system was incubated in shaking condition at 160 rpm, 40 °C up to 60 h. Chemical saccharification of the 2% agro waste biomass was carried out with 1N HCl for comparison. After 12 and 60 h of incubation, samples were withdrawn and estimated for released reducing sugar by DNS method. 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 particular cocktail.

3.2.3.1. Estimation of reducing sugar during agrowaste saccharification:

Reducing sugar released from the biomass was estimated using DNS reagent. 300 µl di-nitro salicylic acid (DNS) reagent was added to 300 µl of enzyme hydrolysate collected from the agrowaste biomass saccharification assay system, 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 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.

3.2.3.2. Calculation of % Saccharification and Degree of Synergism (DS):

% Saccharification can be defined as the amount of reducing sugar in % released after hydrolysis of total substrate provided.

$$\%\, Saccharification = \frac{released\ reducing\ sugar\ (mg/ml)}{Substrate\ provided\ (mg/ml)} \times 100 \qquad \text{(Eq. 3.1)}$$

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

$$Degree\ of\ synergism = \frac{\%\ saccharification\ by\ enzyme\ cocktail}{Cummulative\ \%\ saccharification\ by\ individual\ enzyme} \qquad \text{(Eq. 3.2)}$$

3.2.4. Structural analysis of raw and enzyme hydrolyzed biomass:

During saccharification studies enzymatic actions alter the structural composition of the plant cell wall. Scanning Electron Microscopy and (SEM) was used for morphological and structural analysis while Fourier-Transform Infrared Spectroscopy (FTIR) was used for chemical analysis of raw and enzymatically saccharified biomass material.

3.2.4.1. Scanning Electron Microscopy (SEM):

The selected plant biomass samples were collected in a microfuge tube and washed with phosphate buffered saline (PBS) pH 7.2 ± 0.2. The sample was 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, the biomass was air dried and placed on an adhesive carbon tape fixed on metal stub, sputter coated with Platinum (Pt) in Auto Fine Coater (JEOL-JFC-1600) and then examined under Scanning Electron Microscope (JEOL, JSM-7600F-FEG-SEM) at 10kV in Sophisticated Analytical Instrument Facility (SAIF), IIT-Powai, Mumbai, India. Electron micrographs were taken at desired magnifications.

3.2.4.2. Fourier Transformed Infrared (FTIR) Spectroscopy:

Fourier Transform Infrared (FTIR) spectroscopy was carried out for both raw and enzymatically hydrolysed 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 ground in mortar pestle and a thin pellet was made with a dye using a hydraulic press. Spectra of FTIR were obtained over the range of 400-4000 cm^{-1} with a spectral resolution of 0.5 cm^{-1} on NICOLET 6700, (Thermo Fisher Sci. Instruments) (Magalhães da Silva et al., 2013; Rajak and Banerjee, 2015).

3.2.5. Data Analysis:

- All the Quantitative estimation experiments were performed in triplicates (n=3) and data and error values are represented as Mean ± Standard Error of Mean (SEM) either in GraphPad Prism 6.0 or Origin 2017 software. Statistical analysis was carried out using Two-way ANOVA method in GraphPad Prism 6.0.

3.3. Results and Discussion:

The isolation of plant cell wall polysaccharide degrading microorganism concluded with selection of three isolates, *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208, capable of producing cellulase free xylanase and pectinase enzymes. Being cellulase free in nature, these crude xylanases and pectinases were used to formulate different enzyme cocktails between them as well as with commercial cellulase enzyme to study their synergism in cocktail mediated enzymatic saccharification of agrowaste biomass.

Different cocktails viz., cellulase-pectinase (CP), cellulase-xylanase (CX), pectinase-xylanase (PX) and cellulase-pectinase-xylanase (CPX) were applied individually on different raw untreated agrowaste biomass substrates to study enzymatic synergism. SCB is a major waste product from sugar industries in India, and as wheat & barley husks are major crop in Gujarat and Maharashtra, they are easily available agrowaste products. The proximate composition of these three agro-waste biomass without any pretreatment in terms of % cellulose, hemicellulose, pectin (Galactan) and lignin has been reported as follows: BH: 34.8, 30.4, 1.1, and 24.5 (Krawczyk et al., 2008); SCB: 41.4, 28.2, 1.3, and 23.6 (Ferreira-Leitão et al., 2010); WH: 36, 18 1.9 and 16 (Bledzki et al., 2010). The differences in composition of structural polysaccharides in these agrowaste residues was one of the reasons for their selection for enzymatic saccharification studies as they can actually help to judge the accessory role of each enzyme in saccharification.

3.3.1. Saccharification of raw agrowaste biomass by individual xylanases, pectinases and commercial cellulase:

The treatment of the selected agro-wastes BH, SCB and WH with individual pectinase and xylanase from individual cultures as well as with commercial cellulase (C) released more reducing sugar as the reaction progressed from 12 h to 60 h as shown in Figure 3.1. WB induced xylanases from the *Bacillus* cultures released equivalent or more reducing sugar than commercial cellulase from all three agrowastes. Accordingly, cellulase released approximately 4.6, 5.0 and 9.6 mg of reducing sugars from BH, SCB and WH respectively which was similar to the amount of sugar released by M35 xylanase (4.1, 5.3 and 9.1 mg) and less than that released by R31 xylanase (7.5, 7.2 and 9.7 mg) and J208 xylanase (8.7, 9.3 and 14.0 mg). Whereas pectinases from individual cultures released lesser reducing sugar than cellulase from all three agrowastes tested.

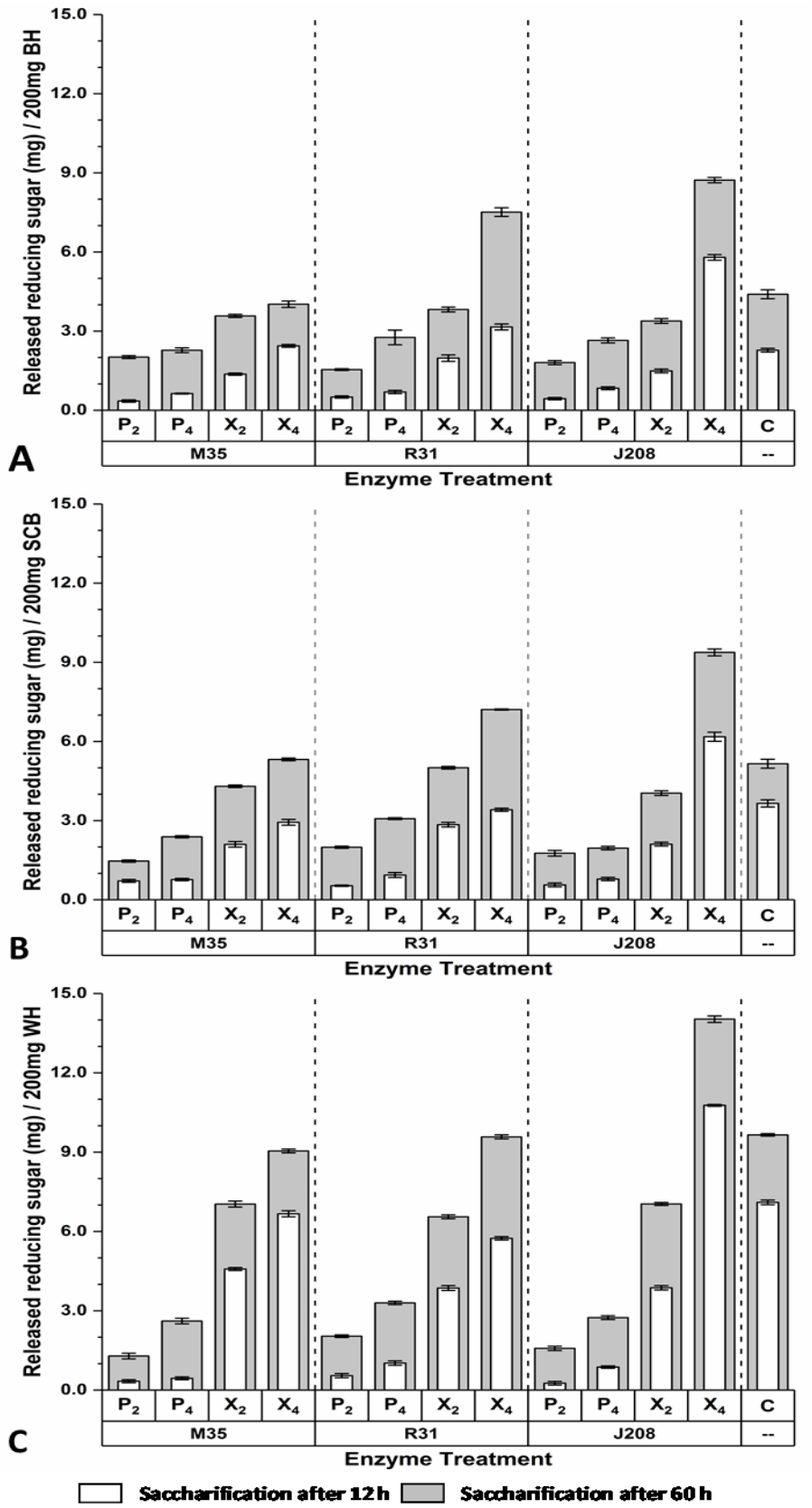

Figure 3.1. Saccharification of raw agrowaste biomass by individual pectinase, xylanase and commercial cellulase:

Saccharification of **(A)** BH, **(B)** SCB and **(C)** WH; P_2, X_2 and P_4, X_4 is 0.2 or 0.4 mg protein equivalent of pectinase and xylanase enzymes; Error bars represented as Standard Errors of the Mean (SEM), for n=3.

When glucan, hemicellulose and galactan (pectin) content in agrowaste composition was correlated with the above observations, it was suggested that reduction in release of the end products by all three pectinases during saccharification of BH, SCB and WH was possibly due to the presence of low amount of structural pectin than xylan or glucan in these agro-wastes. But in spite of higher amount of cellulose than hemicellulose in composition of all three agrowastes, the lesser yield by cellulase than the by the *Bacillus* xylanases was possibly due to the cellulose-hemicellulose meshwork which was impenetrable to commercial cellulase and restricted its action on cellulose.

These observations suggested that the core group of cellulase(s) in the commercial enzyme preparation requires an assistance of accessory enzymes i.e., essentially xylanases and pectinases, to unmask the cellulose fibers from the matrix of hemicellulose and pectin.

3.3.2. Saccharification of raw agrowaste biomass by different cocktail combinations of xylanases, pectinases and commercial cellulase:

In order to analyze the effect of individual xylanase and pectinase supplementation in two enzymes containing CX, CP and PX cocktails and three enzyme CPX cocktails, saccharification of three different agrowaste biomass by these cocktails were analyzed as mentioned below.

3.3.2.1. Saccharification of raw agrowaste biomass by cellulase-xylanase (CX) and cellulase-pectinase (CP) cocktail:

When, two different concentrations of crude xylanases and pectinases separately from individual cultures along with commercial cellulase were used to study the additive synergistic interactions among them, they aided effective saccharification of agrowaste where enhanced % saccharification was observed at 60 h when compared to 12 h.

Incubation of WH with CX cocktail after 60 h practically yielded maximum of 14.71% saccharification with 1.24 fold increase against cumulative value (i.e., theoretically summed value for individual C and X enzyme) of 11.84%. While in case of BH and SCB 10.08 and 8.42% saccharification values with corresponding 1.24 and 1.40 fold increase were obtained by the CX cocktail, against the cumulative values of 7.22 and 6.47 for BH and SCB respectively as shown in Figure 3.2 and Table 3.1.

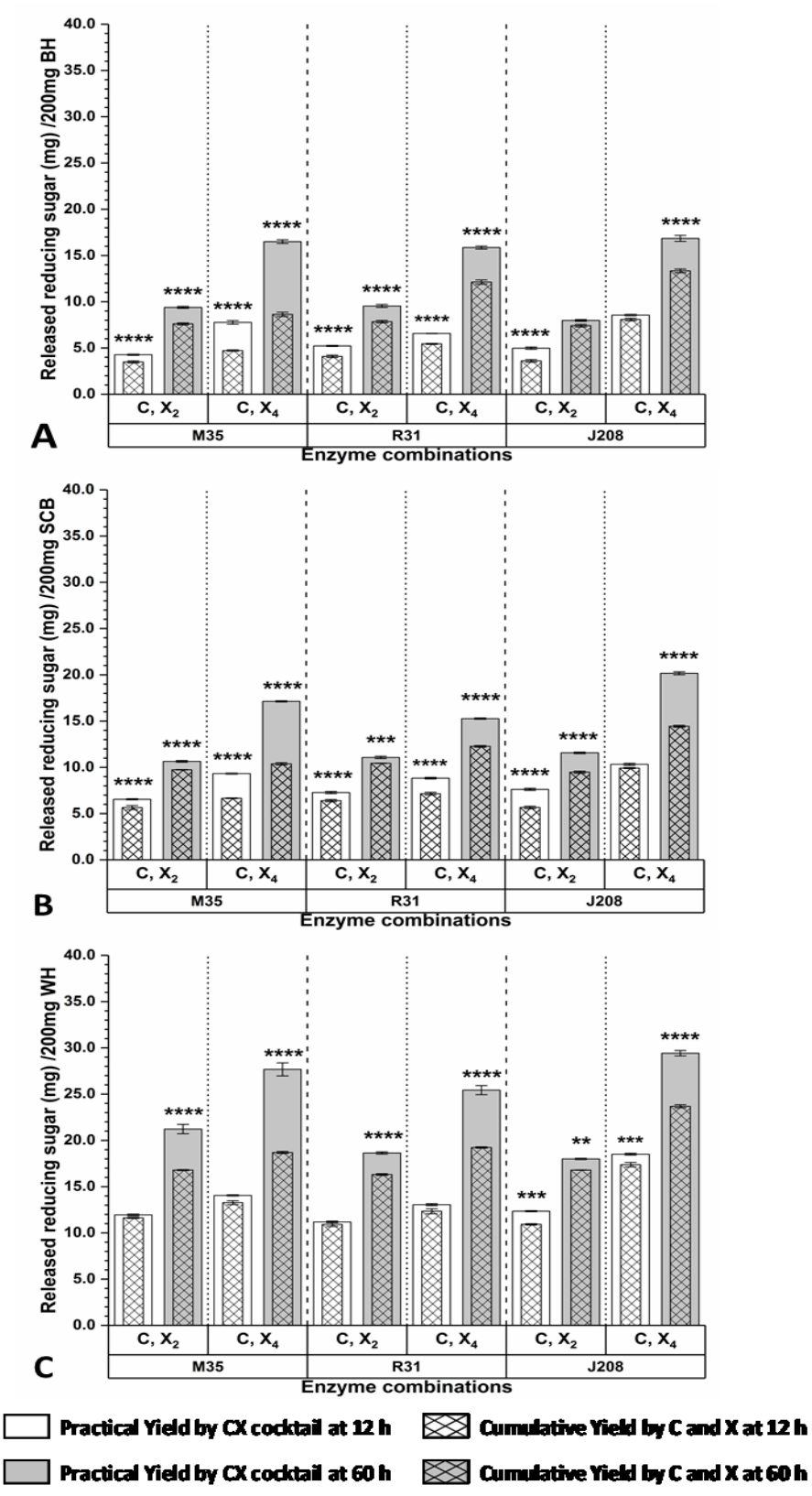

Figure 3.2. Saccharification of raw agrowaste biomass by CX cocktail:
Synergistic effect of commercial cellulase (C) and xylanase (X) combinations from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 on **(A)** BH, **(B)** SCB and **(C)** WH hydrolysis; 0.2 mg and 0.4 mg enzyme load for each culture are grouped for 12 and 60 h; Error bars represented as Standard Errors of the Mean (SEM), Significance difference between PY and CY is given as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** $p < 0.0001$; for n=3.

Table 3.1. % Saccharification and fold increase in % Saccharification (Degree of Synergism, DS) by cellulase and xylanase combinations:

Biomass	Enzyme Combinations	CY		PY		DS	
	Time	12h	60h	12h	60h	12h	60h
Barley Husk (BH)	M35 (C, X_2)	1.74 ± 0.08	3.80 ± 0.08	2.13 ± 0.08	4.69 ± 0.07	1.22	1.23
	M35 (C, X_4)	2.36 ± 0.05	4.32 ± 0.16	3.88 ± 0.05	8.25 ± 0.14	1.64	1.91
	R31 (C, X_2)	2.05 ± 0.09	3.92 ± 0.10	2.61 ± 0.09	4.77 ± 0.11	1.27	1.22
	R31 (C, X_4)	2.72 ± 0.04	6.06 ± 0.16	3.28 ± 0.04	7.93 ± 0.11	1.21	1.31
	J208 (C, X_2)	1.81 ± 0.10	3.71 ± 0.10	2.49 ± 0.10	3.99 ± 0.07	1.38	1.08
	J208 (C, X_4)	4.04 ± 0.10	6.67 ± 0.14	4.28 ± 0.10	8.42 ± 0.23	1.06	1.26
Sugarcane Bagasse (SCB)	M35 (C, X_2)	2.83 ± 0.16	4.87 ± 0.02	3.27 ± 0.16	5.32 ± 0.07	1.16	1.09
	M35 (C, X_4)	3.33 ± 0.03	5.19 ± 0.09	4.66 ± 0.33	8.56 ± 0.05	1.40	1.65
	R31 (C, X_2)	3.20 ± 0.09	5.22 ± 0.02	3.63 ± 0.09	5.54 ± 0.09	1.14	1.06
	R31 (C, X_4)	3.58 ± 0.10	6.13 ± 0.06	4.41 ± 0.10	7.63 ± 0.05	1.23	1.24
	J208 (C, X_2)	2.83 ± 0.10	4.74 ± 0.08	3.81 ± 0.10	5.78 ± 0.06	1.34	1.22
	J208 (C, X_4)	4.96 ± 0.04	7.22 ± 0.07	5.16 ± 0.04	10.08 ± 0.11	1.04	1.40
Wheat Husk (WH)	M35 (C, X_2)	5.82 ± 0.05	8.40 ± 0.04	5.97 ± 0.05	10.61 ± 0.36	1.03	1.26
	M35 (C, X_4)	6.64 ± 0.15	9.35 ± 0.08	7.03 ± 0.15	13.84 ± 0.50	1.06	1.48
	R31 (C, X_2)	5.46 ± 0.15	8.16 ± 0.07	5.59 ± 0.15	9.32 ± 0.09	1.02	1.14
	R31 (C, X_4)	6.17 ± 0.19	9.61 ± 0.05	6.53 ± 0.19	12.72 ± 0.35	1.06	1.32
	J208 (C, X_2)	5.46 ± 0.05	8.40 ± 0.01	6.17 ± 0.05	9.00 ± 0.05	1.13	1.07
	J208 (C, X_4)	8.69 ± 0.16	11.84 ± 0.12	9.25 ±0.16	14.71 ± 0.21	1.07	1.24

CY: Cumulative Yield; PY: Practical Yield; DS: Degree of Synergism; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

As shown in Figure 3.3 and Table 3.2, the CP cocktail after 60 h of incubation, brought about 11.72% saccharification with 1.90 fold increase against cumulative value of 6.20 by individual enzymes on WH. Whereas, 6.45 and 4.82% saccharification values with corresponding 1.78 and 1.37 fold increase were obtained by the CP cocktail against the cumulative values of 3.63 and 3.51 respectively for BH and SCB.

In all three CX cocktail combinations of cellulase-xylanase and three PX cocktail combinations of cellulase-pectinase, synergism was observed as the endproducts released by CP or CX cocktail (CP_2, CP_4, CX_2 and CX_4), exceeded than the cumulative values (or theoretical sum, $C+P_2$, $C+P_4$, $C+X_2$ and $C+X_4$) of the endproducts released by the individual enzyme when used separately, and their ratio was higher than 1 indicating enhancement in saccharification. In case of pectinase containing cocktail, increased dosage of pectinase (0.4 mg instead of 0.2 mg) in some cases were observed to yield less saccharification during 12 h incubation possibly due to the structural complexities of the agrowaste substrates.

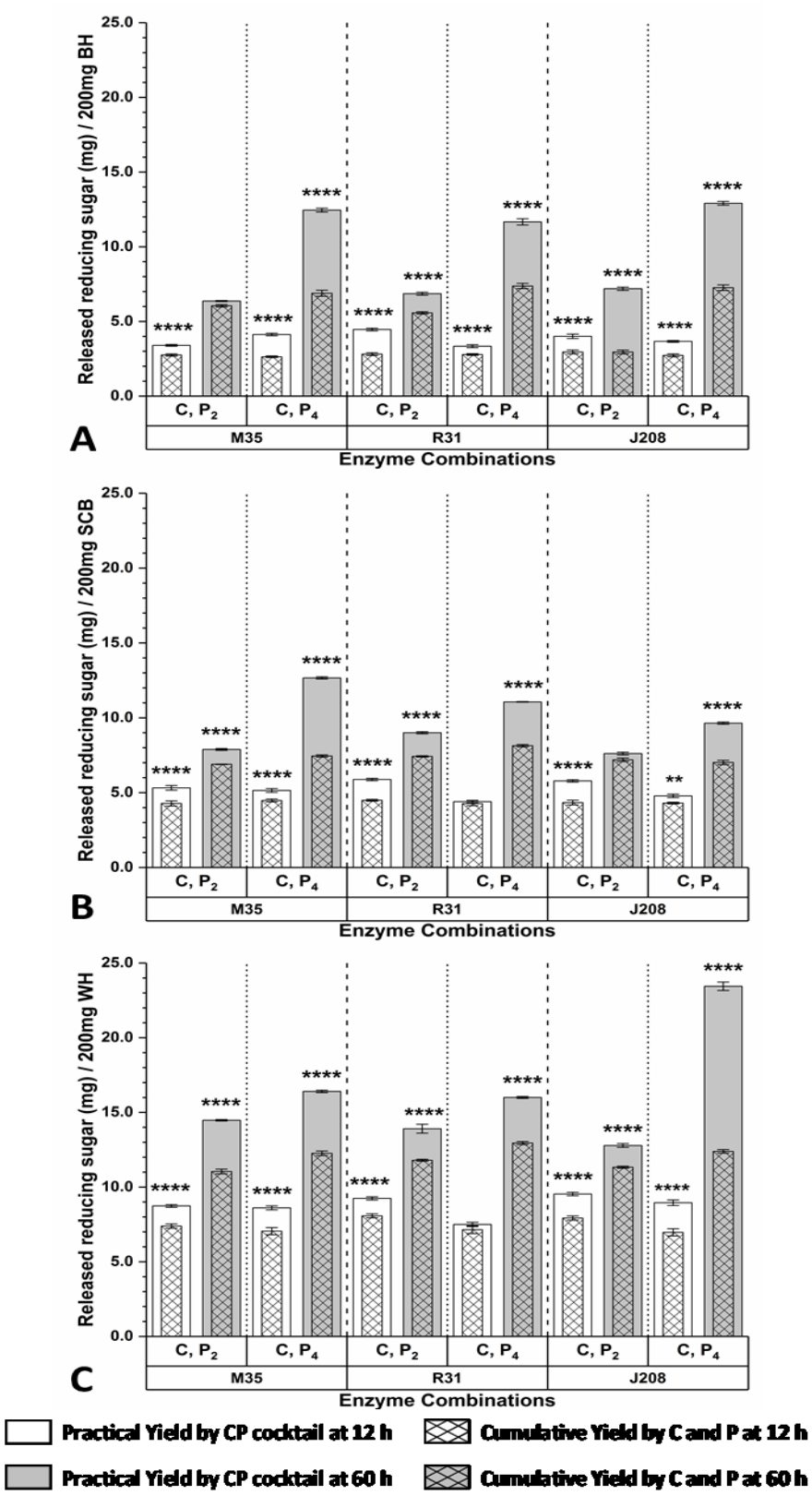

Figure 3.3. Saccharification of raw agrowaste biomass by CP cocktail:
Synergistic effect of commercial cellulase (C) and pectinase (P) combinations from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 on **(A)** BH, **(B)** SCB and **(C)** WH hydrolysis; 0.2 mg and 0.4 mg enzyme load for each culture are grouped for 12 and 60 h; Error bars represented as Standard Errors of the Mean (SEM); Significance difference between PY and CY is given as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** $p < 0.0001$; for n=3.

Table 3.2. % Saccharification and fold increase in % Saccharification (Degree of Synergism, DS) by cellulase and pectinase combinations:

Biomass	Enzyme Combinations	CY		PY		DS	
	Time	12h	60h	12h	60h	12h	60h
Barley Husk (BH)	M35 (C, P_2)	1.38 ± 0.05	3.02 ± 0.05	1.70 ± 0.04	3.18 ± 0.02	1.24	1.05
	M35 (C, P_4)	1.32 ± 0.04	3.44 ± 0.14	2.06 ± 0.06	6.22 ± 0.09	1.57	1.81
	R31 (C, P_2)	1.41 ± 0.07	2.79 ± 0.05	2.23 ± 0.06	3.43 ± 0.07	1.59	1.23
	R31 (C, P_4)	1.39 ± 0.04	3.69 ± 0.12	1.67 ± 0.07	5.83 ± 0.15	1.20	1.58
	J208 (C, p_2)	1.48 ± 0.09	2.92 ± 0.08	2.00 ± 0.11	3.59 ± 0.08	1.36	1.23
	J208 (C, P_4)	1.36 ± 0.07	3.63 ± 0.13	1.83 ± 0.04	6.45 ± 0.09	1.35	1.78
Sugarcane Bagasse (SCB)	M35 (C, P_2)	2.14 ± 0.12	3.45 ± 0.01	2.66 ± 0.11	3.94 ± 0.04	1.25	1.14
	M35 (C, P_4)	2.24 ± 0.07	3.72 ± 0.05	2.57 ± 0.09	6.33 ± 0.05	1.15	1.70
	R31 (C, P_2)	2.24 ± 0.04	3.71 ± 0.03	2.94 ± 0.06	4.50 ± 0.05	1.31	1.21
	R31 (C, P_4)	2.14 ± 0.10	4.06 ± 0.06	2.20 ± 0.06	5.53 ± 0.02	1.03	1.36
	J208 (C, P_2)	2.17 ± 0.11	3.60 ± 0.08	2.89 ± 0.06	3.81 ± 0.07	1.34	1.06
	J208 (C, P_4)	2.15 ± 0.04	3.51 ± 0.10	2.39 ± 0.08	4.82 ± 0.05	1.11	1.37
Wheat Husk (WH)	M35 (C, P_2)	3.69 ± 0.10	5.52 ± 0.11	4.37 ± 0.07	7.23 ± 0.03	1.18	1.31
	M35 (C, P_4)	3.52 ± 0.17	6.13 0.10	4.30 ± 0.09	8.20 ± 0.06	1.22	1.34
	R31 (C, P_2)	4.04 ± 0.10	5.90 ± 0.05	4.62 ± 0.07	6.95 ± 0.21	1.15	1.18
	R31 (C, P_4)	3.58 ± 0.19	6.47 ± 0.07	3.75 ± 0.10	8.00 ± 0.04	1.05	1.24
	J208 (C, P_2)	3.96 ± 0.10	5.67 ± 0.05	4.77 ± 0.08	6.39 ± 0.09	1.20	1.13
	J208 (C, P_4)	3.48 ± 0.17	6.20 ± 0.08	4.48 ± 0.13	11.72 ± 0.20	1.29	1.90

CY: Cumulative Yield; PY: Practical Yield; DS: Degree of Synergism; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

During the initial 12 h period of incubation, the amount of pectin available was less but as the incubation time increased to 60 h more pectin was available due to disintegration of the cell wall complex and therefore the pectinase demonstrated synergism with the other enzymes for enhanced saccharification. Zhang et al., (2013) has reported 1.3 and 1.2 fold increase in % saccharification by xylanase and pectinase respectively during synergism studies with fungal cellulase. While Li et al., (2014) used NaOH pretreated, H_2O_2 pretreated & steam exploded sugarcane bagasse and exhibited up to 1.6 fold increase in saccharification due to synergism between fungal endo-1,4-β-xylanase with Celluclast 1.5 L enzymes.

These results suggested that, the xylanase and pectinase from *B. safensis* M35, B. *altitudinis* R31 and *B. altitudinis* J208 individually exhibited synergism with cellulase and played accessory role in cellulase mediated saccharification of three different agrowastes WH, BH and SCB. So, prior to perform further studies regarding formation of CPX cocktail, it was necessary to study the compatibility of these

accessory xylanase and pectinase enzymes with each-other.

3.3.2.2. Saccharification of agrowaste biomass by pectinase-xylanase (PX) cocktail:

Compatibility of xylanases and pectinases from same culture with each other brought about effective % saccharification of agrowaste after 60 h. PX cocktail after 60 h exhibited 9.88 % saccharification with 1.18 fold increase against cumulative value of 8.38 due to individual enzymes on WH. Whereas, 9.37 and 6.69 were % saccharification values with corresponding 1.65 and 1.18 fold increase obtained by the PX cocktail against the cumulative values of 5.69 and 5.67 for BH and SCB respectively as shown in Figure 3.4 and Table 3.3. Positive values of fold increase by PX cocktail indicated their compatibilities in synergism with each other.

While, the three enzyme combinations CX, CP and PX showed increase in reducing sugar released, maximum release of sugar was observed generally in case of CX followed by CP and PX at 60 h on all three substrates.

Table 3.3. % Saccharification and fold increase in % Saccharification (Degree of Synergism, DS) by pectinase and xylanase combinations:

Biomass	Enzyme Combinations	CY		PY		DS	
	Time	12h	60h	12h	60h	12h	60h
Barley Husk (BH)	M35 (P_2, X_2)	1.00 ± 0.04	2.80 ± 0.06	1.32 ± 0.04	3.03 ± 0.05	1.32	1.08
	M35 (P_4, X_4)	1.40 ± 0.07	3.15 ± 0.14	1.53 ± 0.08	5.49 ± 0.06	1.0	1.74
	R31 (P_2, X_2)	1.34 ± 0.13	2.68 ± 0.08	1.61 ± 0.05	2.95 ± 0.05	1.22	1.10
	R31 (P_4, X_4)	1.83 ± 0.11	5.14 ± 0.23	1.92 ± 0.04	6.69 ± 0.06	1.05	1.31
	J208 (P_2, X_2)	1.16 ± 0.09	2.60 ± 0.10	1.59 ± 0.04	2.91 ± 0.04	1.38	1.12
	J208 (P_4, X_4)	3.12 ± 0.06	5.69 ± 0.10	3.65 ± 0.07	9.37 ± 0.10	1.17	1.65
Sugarcane Bagasse (SCB)	M35 (P_2, X_2)	1.41 ± 0.11	2.88 ± 0.06	1.70 ± 0.04	3.43 ± 0.07	1.21	1.19
	M35 (P_4, X_4)	1.85 ± 0.10	3.85 ± 0.05	2.13 ± 0.05	4.78 ± 0.04	1.16	1.24
	R31 (P_2, X_2)	1.89 ± 0.12	3.50 ± 0.05	2.34 ± 0.08	3.98 ± 0.08	1.24	1.13
	R31 (P_4, X_4)	1.97 ± 0.05	5.14 ± 0.01	2.03 ± 0.04	5.41 ± 0.06	1.03	1.05
	J208 (P_2, X_2)	1.45 ± 0.09	2.90 ± 0.09	1.92 ± 0.12	3.39 ± 0.04	1.34	1.16
	J208 (P_4, X_4)	3.12 ± 0.06	5.67 ± 0.10	3.65 ± 0.07	6.69 ± 0.04	1.12	1.18
Wheat Husk (WH)	M35 (P_2, X_2)	2.46 ± 0.04	4.16 ± 0.05	2.96 ± 0.06	4.39 ± 0.07	1.20	1.05
	M35 (P_4, X_4)	3.56 ± 0.11	5.83 ± 0.11	3.86 ± 0.16	6.92 ± 0.10	1.09	1.19
	R31 (P_2, X_2)	2.44 ± 0.09	4.30 ± 0.09	2.88 ± 0.04	4.53 ± 0.05	1.18	1.05
	R31 (P_4, X_4)	3.14 ± 0.09	6.44 ± 0.08	3.24 ± 0.06	6.55 ± 0.05	1.03	1.02
	J208 (P_2, X_2)	2.37 ± 0.07	4.31 ± 0.08	2.89 ± 0.10	4.65 ± 0.08	1.22	1.08
	J208 (P_4, X_4)	5.57 ± 0.05	8.38 ± 0.14	6.21 ± 0.06	9.88 ± 0.45	1.12	1.18

CY: Cumulative Yield; PY: Practical Yield; DS: Degree of Synergism; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

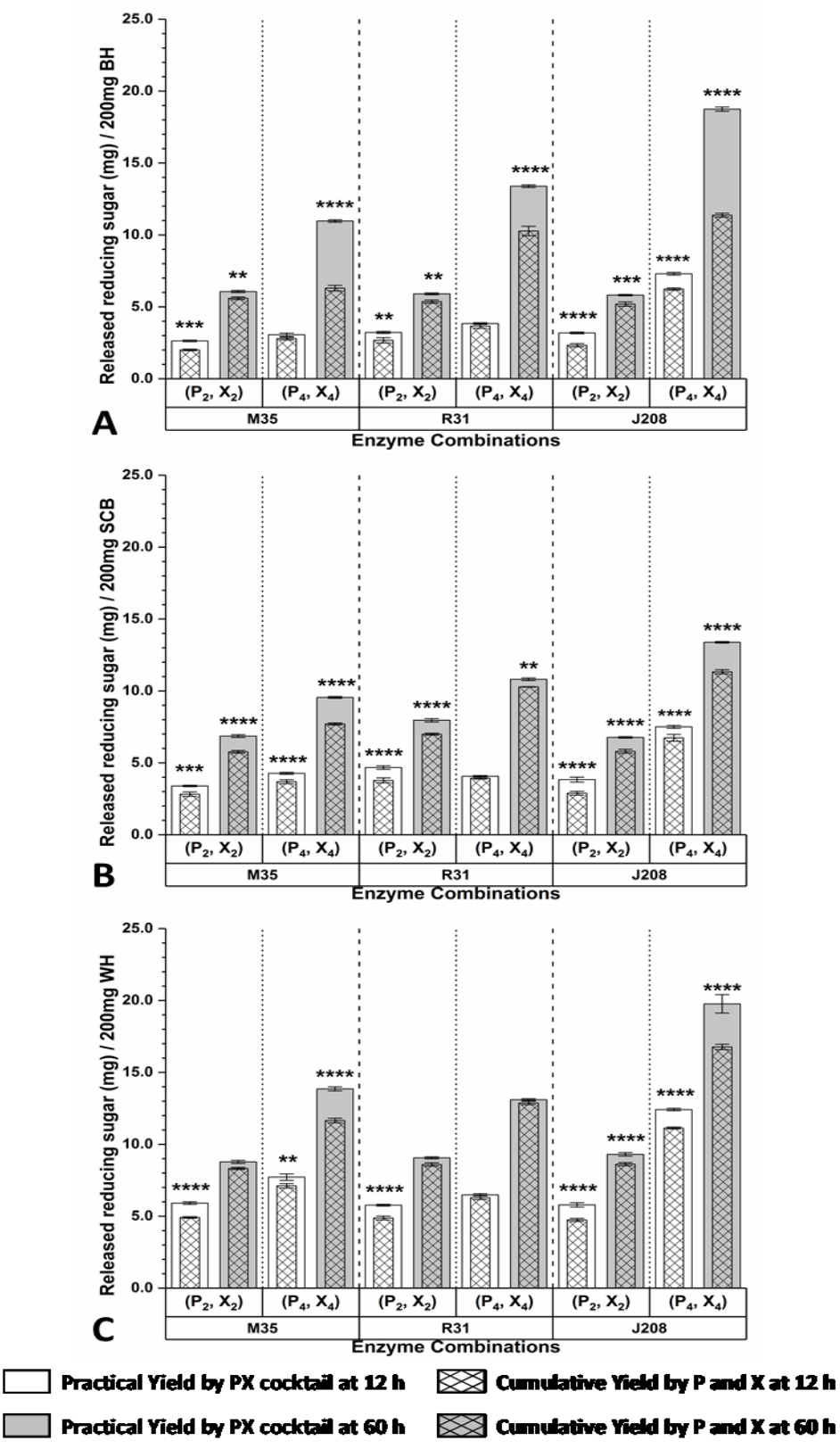

Figure 3.4. Saccharification of raw agrowaste biomass by PX cocktail:
Synergistic effect of pectinase (P) and xylanase (X) combinations from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 on **(A)** BH, **(B)** SCB and **(C)** WH hydrolysis; 0.2 mg and 0.4 mg enzyme load for each culture are grouped for 12 and 60 h; Error bars represented as Standard Errors of the Mean (SEM); Significance difference between PY and CY is given as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** $p < 0.0001$; for n=3.

These results opened a room for xylanases and pectinases from *B. safensis* and *B. altitudinis* isolates for their use as accessory enzymes in combined application with commercial cellulase to form an efficient CPX cocktail for biomass saccharification application.

3.3.2.3. Saccharification of biomass by three enzyme cocktail CPX:

Three different CPX cocktails were formulated using xylanase and pectinase from individual cultures with commercial cellulase for saccharification studies of three untreated biomass substrates BH, SCB and WB. CP_4X_4 cocktail hydrolysed the BH, SCB and WH to maximum values of 10.58, 9.68 and 16.14 % saccharification against the cumulative values of 7.99, 8.20 and 13.21% during incubation up to 60 h with fold increase of 1.32, 1.18 and 1.22. With supplementation of higher dosage i.e., 0.4 mg when compared to 0.2 mg of enzymes with fixed amount of cellulase, the hydrolysis yields of agro-wastes eventually increased and resulted in higher positive fold increase of % saccharification gradually with time as shown in Figure 3.5 and Table 3.4.

Table 3.4. % Saccharification and fold increase in % Saccharification (Degree of Synergism, DS) by cellulase, pectinase and xylanase combinations:

Biomass	Enzyme Combination	CY		PY		DS	
	Time	12h	60h	12h	60h	12h	60h
Barley Husk (BH)	M35 (C, P_2, X_2)	2.06 ± 0.08	4.81 ± 0.09	2.43 ± 0.08	5.04 ± 0.10	1.18	1.05
	M35 (C, P_4, X_4)	2.54 ±0.06	5.46 ± 0.22	3.49 ± 0.09	9.10 ± 0.02	1.38	1.67
	R31 (C, P_2, X_2)	2.39 ± 0.14	4.69 ± 0.17	2.82 ± 0.03	4.96 ± 0.06	1.18	1.06
	R31 (C, P_4, X_4)	2.97 ±0.07	7.44 ± 0.20	3.49 ± 0.09	10.08 ± 0.40	1.18	1.35
	J208 (C, P_2, X_2)	2.22 ± 0.14	4.61 ± 0.14	2.87 ± 0.09	5.00 ± 0.04	1.29	1.09
	J208 (C, P_4, X_4)	4.26 ±0.10	7.99 ± 0.18	4.58 ± 0.05	10.58 ± 0.20	1.08	1.32
Sugarcane Bagasse (SCB)	M35 (C, P_2, X_2)	3.19 ± 0.19	5.60 ± 0.05	3.81 ± 0.11	5.78 ± 0.04	1.20	1.03
	M35 (C, P_4, X_4)	3.71 ±0.05	6.38 ± 0.09	3.89 ± 0.09	8.13 ± 0.08	1.05	1.27
	R31 (C, P_2, X_2)	3.67 ± 0.09	6.21 ± 0.03	4.36 ± 0.08	7.27 ± 0.07	1.19	1.17
	R31 (C, P_4, X_4)	3.84 ± 0.12	7.67 ± 0.050	4.07 ± 0.08	7.84 ± 0.08	1.06	1.02
	J208 (C, P_2, X_2)	3.22 ± 0.14	5.62 ± 0.12	3.77 ± 0.07	6.97 ± 0.08	1.17	1.24
	J208 (C, P_4, X_4)	5.24 ± 0.09	8.20 ± 0.11	5.55 ± 0.14	9.68 ± 0.12	1.06	1.18
Wheat Husk (WH)	M35 (C, P_2, X_2)	5.98 ± 0.06	9.04 ± 0.06	6.97 ± 0.15	9.86 ± 0.44	1.16	1.09
	M35 (C, P_4, X_4)	6.86 ± 0.18	10.66 ± 0.15	7.09 ± 0.06	12.10 ± 0.08	1.03	1.14
	R31 (C, P_2, X_2)	5.97 ± 0.16	9.17 ± 0.10	6.69 ± 0.07	9.83 ± 0.15	1.12	1.07
	R31 (C, P_4, X_4)	6.44 ± 0.23	11.26 ± 0.09	6.87 ± 0.05	12.31 ± 0.30	1.07	1.10
	J208 (C, P_2, X_2)	5.90 ± 0.08	9.19 ± 0.05	6.45 ± 0.15	10.45 ± 0.05	1.09	1.14
	J208 (C, P_4, X_4)	8.87 ± 0.19	13.21 ± 0.17	9.58 ± 0.11	16.14 ± 0.22	1.08	1.22

CY: Cumulative Yield; PY: Practical Yield; DS: Degree of Synergism; Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

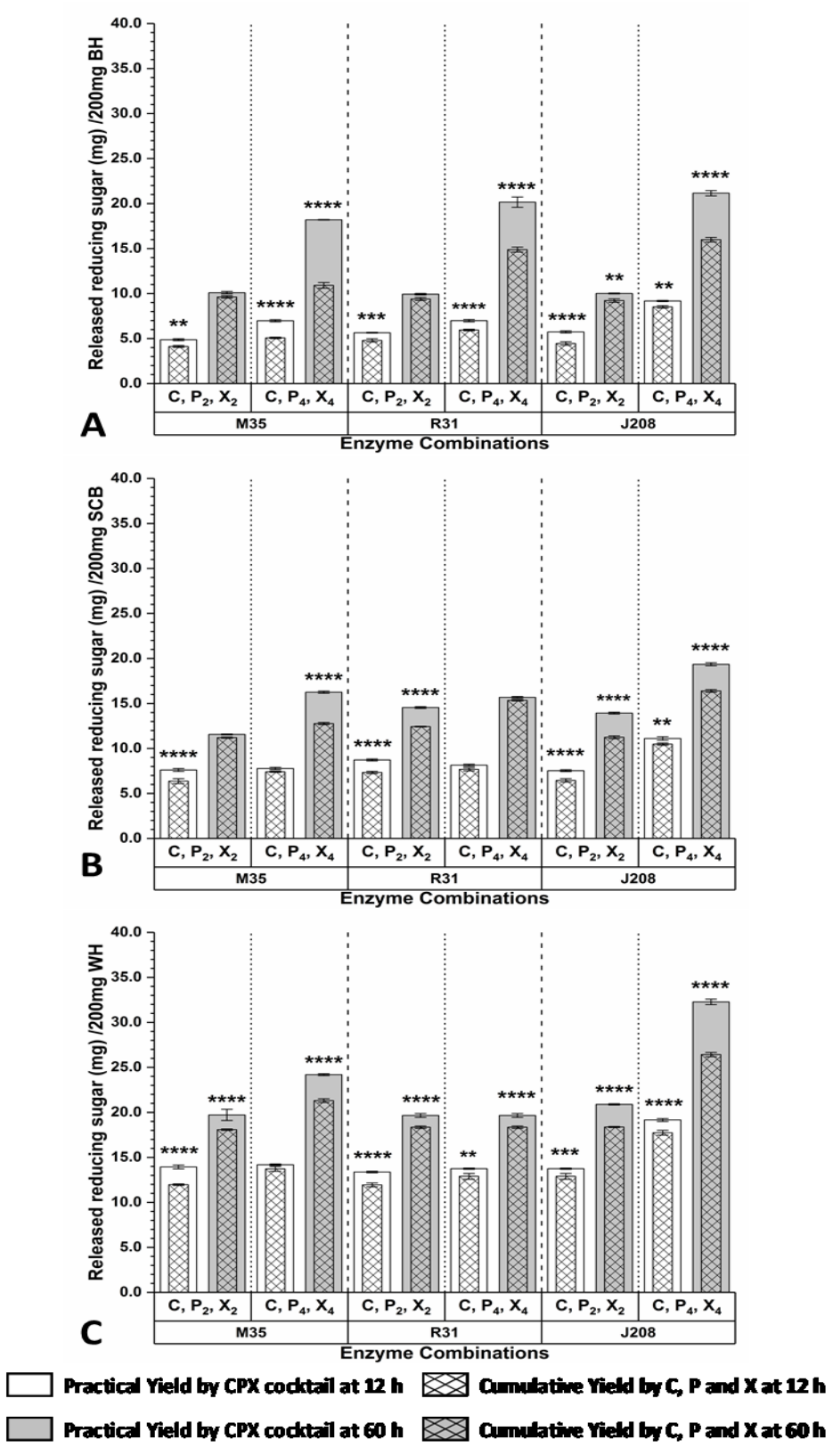

Figure 3.5. Saccharification of raw agrowaste biomass by CPX cocktail:
Synergistic effect of pectinase (P) and xylanase (X) combinations from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 with commercial cellulase on **(A)** BH, **(B)** SCB and **(C)** WH hydrolysis; 0.2 mg and 0.4 mg enzyme load for each culture are grouped for 12 and 60 h; Error bars represented as Standard Errors of the Mean (SEM); Significance difference between PY and CY is given as * = p <0.05, ** = p <0.01, *** = p <0.001 and **** p <0.0001; for n=3.

During saccharification studies by CPX cocktail through additive approach xylanase and pectinase from individual isolates exhibited synergism with commercial cellulase as released endproducts by cocktail (CP_2X_2 and CP_4X_4), exceeded the cumulative values (or sum) of the endproducts released by the individual enzymes when used separately ($C+P_2+X_2$ and $C+P_4+X_4$) and their ratio was higher than 1 indicating enhancement in saccharification of biomass by cocktail.

3.3.3. Saccharification of agrowaste biomass by 1N HCl:

When efficacies of the different CPX cocktails were compared with the % saccharification values observed in Table 3.5 by chemical method (acid hydrolysis by 1N HCl), enhanced % saccharification of SCB and WH was observed at 60 h. This again suggests that further modification and optimization of cocktail by changing the proportions of different enzyme loadings can lead to enhanced saccharification of biomass in comparison to the chemical method used for saccharification.

Table 3.5. % Saccharification of agrowaste biomass with 1N HCl:

Substrates	Time	12h	60h
	Treatment	% Saccharification	
Barley Husk (BH)	1N HCl	3.60 ± 0.06	14.94 ± 0.36
Sugarcane Bagasse (SCB)		0.74 ± 0.03	7.69 ± 0.27
Wheat Husk (WH)		2.41 ± 0.07	10.14 ± 0.20

Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

Though Primafast®200 showed xylanase activity, supplementation of cellulase free xylano-pectinolytic enzymes obtained individually from isolate *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 enhanced the saccharification suggesting that the pectinase and xylanase both play an important role as accessory enzymes in synergism with commercial cellulase. In similar studies with fresh, steam exploded and ensiled hemp, when accessory xylanase and pectinase of different fungal origin were applied with core cellulase (containing endoglucanase II, cellobiohydrolase I and β-glucosidase), released approximately 5.0, 11.5 and 7.0 mg/ml of reducing sugars after 48 h suggesting synergism of pectinase with xylanase and/or cellulase (Zhang et al., 2013). While xylanase, pectinase and cellulase obtained from *Chrysoporthae cubensis* enhanced saccharification of pretreated SCB (Maitan-Alfenas et al., 2015b).

3.3.4. Structural analysis of raw BH, SCB and WH treated with a cocktail containing commercial cellulase and/or xylanase and pectinase enzymes from *B. altitudinis* J208:

Three raw substrates BH, SCB and WH individually without any enzymatic treatment as well as treated with either only commercial cellulase or J208-CPX cocktail containing both xylanase-pectinase only from *B. altitudinis* J208 were used for SEM analysis to study morphological changes in the structure of cell walls and for FTIR analysis to study the chemical changes in composition of cell walls.

3.3.4.1. Scanning Electron Microscopy (SEM) analysis of raw biomass before and after enzymatic hydrolysis:

Following saccharification, the residual biomass particles were analyzed in longitudinal orientation with SEM. It can be seen from the micrographs in the Figures 3.6 (BH), 3.7 (SCB) and 3.8 (WH), the untreated cell wall images clearly appear intact as compared to cellulase treated and CPX cocktail treated ones. The CPX cocktail treated particles (in image C) as compared to cellulase treated particles (in image B) were showing more damage giving flaky appearance to the cell wall (indicated by the red arrow) suggesting enhanced biomass deconstruction by CPX cocktail than only cellulase. Mukhopadhyay et al., (2011) has reported such flaky appearance of *Ricinus communis* particles after enzymatic treatment of delignified biomass. Kuila et al., (2011) have demonstrated severe structural breakdown of the *Lantana camara* after delignification and saccharification of particulate biomass. The area of intact surface is decreased with increased roughness of surface as well as edges (indicated by blue arrow) from untreated (Image A), cellulase treated (Image B) and CPX cocktail treated (Image C) in this order for each substrate. The enzymatic treatment of the agrowaste substrates with CPX cocktail would possibly have deconstructed or in other words have broken down the complex of cellulose-hemicellulose network present in cell wall matrix and the cellulose would have become more accessible to the cellulase in cell wall. Rajak and Banerjee, (2016) have demonstrated such surface roughness from the Kans grass (*Saccharum spontaneum*) after delignification and saccharification treatment. Thus, the CPX cocktail treatment was more efficient than the only cellulase treatment as, it released more reducing sugars as well as exhibited more breakage to the cell wall structure. To confirm further untreated and enzyme treated particles were subjected to- FTIR analysis.

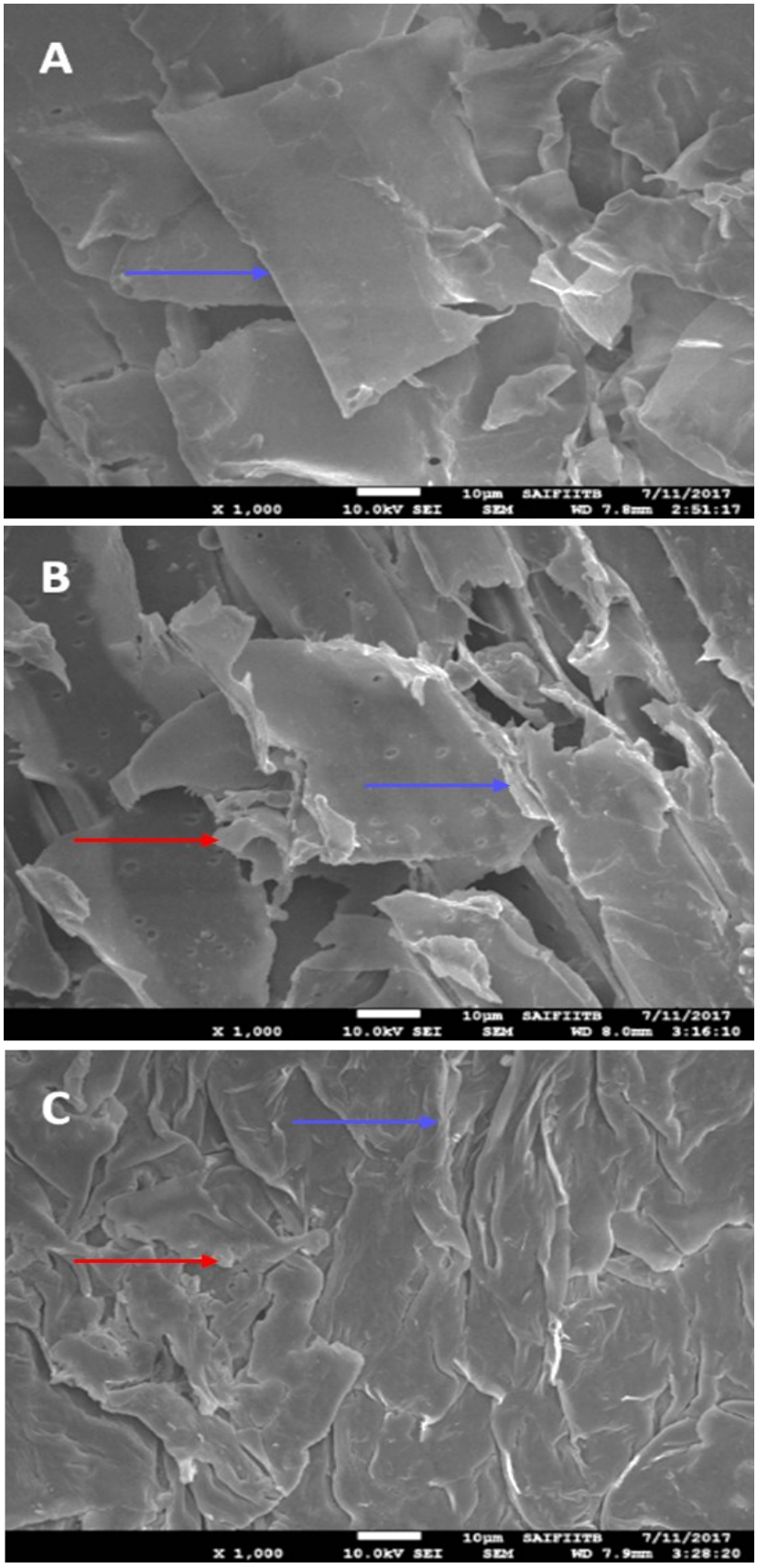

Figure 3.6. Scanning electron micrograph of raw Barley husk:
(A) Untreated control **(B)** Cellulase treated, **(C)** CPX cocktail treated Barley Husk fragments observed at 1000X magnification, 10.0kV, White bar represents scale of 10 µm; Red arrow indicates flakey appearance; Blue arraow: indicates roughness in cell wall edges.

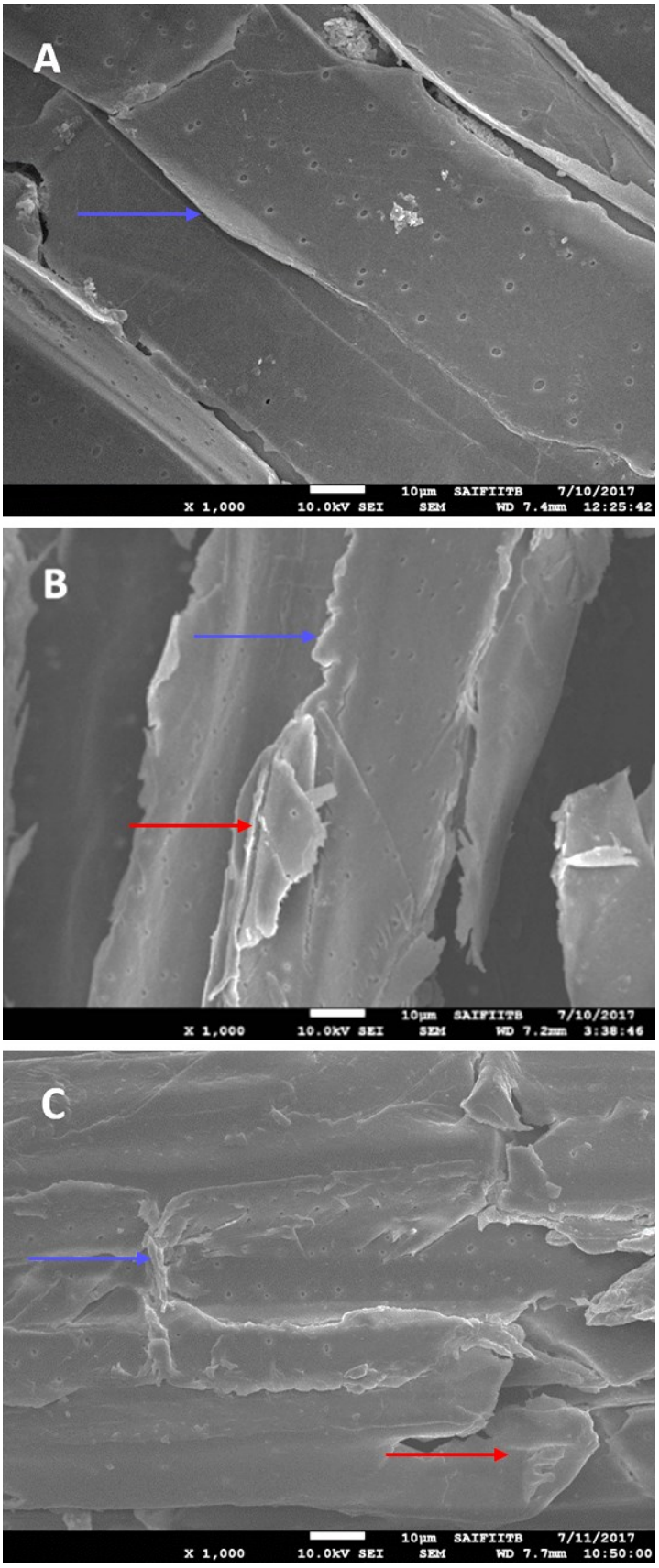

Figure 3.7. Scanning electron micrograph of raw Sugarcane bagasse:
(A) Untreated control **(B)** Cellulase treated, **(C)** CPX cocktail treated Sugarcane Bagasse fragments observed at 1000X magnification, 10.0kV, White bar represents scale of 10 µm; Red arrow indicates flakey appearance; Blue arraow: indicates roughness in cell wall edges.

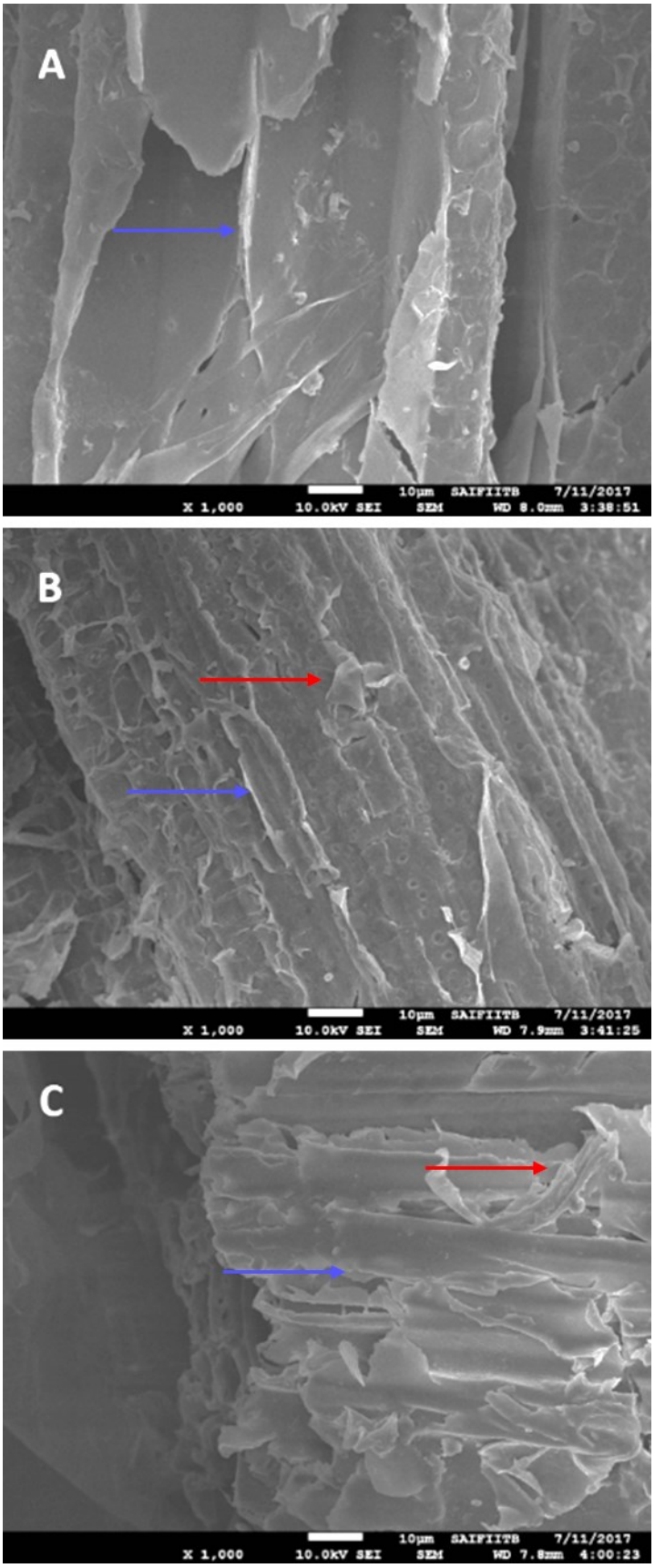

Figure 3.8. Scanning electron micrograph of raw Wheat Husk:
(A) Untreated control **(B)** Cellulase treated, **(C)** CPX cocktail treated Wheat Husk fragments observed at 1000X magnification, 10.0kV, White bar represents scale of 10 µm; Red arrow indicates flakey appearance; Blue arraow: indicates roughness in cell wall edges.

3.3.4.2. Fourier Transformed Infrared (FTIR) spectroscopic analysis of raw and enzymatically treated substrate biomass:

The peaks observed under FTIR spectra and the attributes of the lignocellulosic components to which they are assigned are listed in Table 3.6. as below. Figure 3.9 represents the FTIR spectral profile of each of the three raw substrates before any enzymatic treatment and after saccharification with commercial cellulase, and CP_4X_4 cocktail made up of commercial cellulase and xylano-pectinolytic enzymes from *B. altitudinis* J208.

Table 3.6. FTIR absorbance bands observed for plant biomass in range of wavenumber 400-1800 $cm^{-1}$and the functional groups or bands to which they are attributed:

Wavenumbers (cm^{-1})	The attributions of the main absorptions
1734	Ester cross-linkage between acetyl group of hemicellulose and lignin components like, p-coumaryl, coniferyl or sinapyl alcohol
1630	C=O stretching in conjugated p-substituted aryl ketones
1600, 1510	Aromatic skeleton (C-C) breathing of mono or disubstituted ring and C=O stretching
1430	Crystalline structure of cellulose
1376	C-H stretching due to glycosidic bond vibrations of cellulose
1320	C-C and C-O skeletal stretching vibrations, CH rocking vibrations for glucose ring.
1270	Aromatic ring breathing with C=O stretching
1120	C-OH skeletal vibrations,
1160	Anti-symmetric stretching C—O—C glycoside; C—O—C bond at β-1,4 glycosyl linkage of cellulose-hemicellulose
1070	C=O deformations of 2° alcohol with aliphatic ethers
1060	C– O–C ether linkage of skeletal vibration
1043	glycosidic linkage C–O–C in xylan
1030	Aromatic C–H in plane deformation(G[S), methoxyl group deformation, secondary alcohols, and C=O stretching nonconjugated
997	Arabinose side chains present in hemicellulose
897	Amorphous cellulose vibration; C-H deformations at -C-O-C- β-glycosidic linkages present in pyranose ring of cellulose and hemicelluloses
833	C-H out of plane deformation in p-hydroxyphenyl aromatic rings

Strong and broad absorbance observed at range of 3450-3300 cm^{-1} is an attribute of stretching exhibited by O-H bond of hydroxyl groups which participates in intra as well as intermolecular hydrogen bonding during polymeric association of cellulosic and hemicellulosic polysaccharides (Yang et al., 2007).

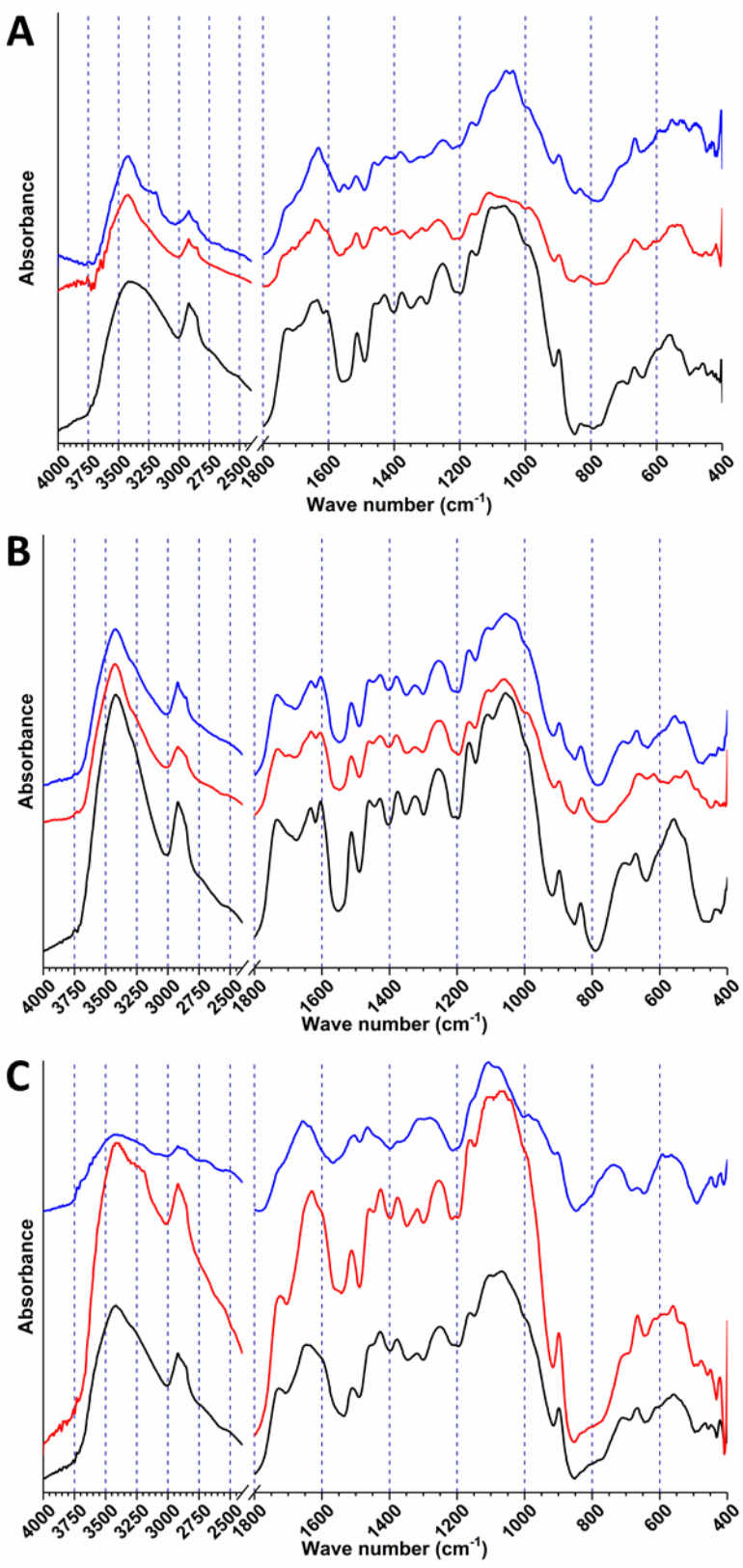

Figure 3.9. FTIR spectra of raw, commercial cellulase treated and J208 CPX cocktail treated agrowaste biomass:
(A) Barley Husk, **(B)** Sugarcane Bagasse and **(C)** Wheat Husk, before any enzymatic treatment (black, ▬), after treatment up to 60 h with commercial cellulase (red, ▬) and CPX cocktail (blue, ▬) at wavenumbers of 400-4000 cm^{-1}; Y-axis is presented as an offset.

The absorbance peaks observed at 2950-2820 cm^{-1} are attributed to -C-H stretching due to symmetrical and asymmetrical stretching vibration for $-CH_3$, $-CH_2$ or -CH group for carbon chain containing compound. Which can be correlated with individual polysaccharide monomers of where -C-H bond can be considered as a specific characteristics of aldehyde carbon (-CHO) while keto (-CO-) carbon lack it.

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). (Silva et al., 2017). Besides these two major stretches, other absorbance patterns in the FTIR spectra were concentrated for wavenumbers 2000 to1000 cm^{-1} which comprise bands assigned to the major components of the biomass, i.e., cellulose, hemicelluloses and lignin. Upadhyay et al., (2009) and Wade, (2013) have explained the detailed characteristic infrared absorptions attributed to several functional groups in the spectra. Since cellulose and hemicellulose are the major components, a stretch of 1750-1705 cm^{-1} representing the C=O stretching due to aliphatic, saturated aldehyde and aliphatic saturated keto groups of sugars were observed. This stretch for individual biomass substrates decreased in declining order WH, BH, and SCB for CPX and cellulase treated biomass relative to untreated biomass. 1250-850 cm^{-1} is the spectra where characteristic vibration bonds of cellulose can be seen. Vibration bands at 897, 1061 cm^{-1} and 1161 cm^{-1} were present in the biomass samples, with and without enzymatic hydrolysis but the cellulase treated and CPX treated samples were with decreased intensities of the peaks. Moreover, a vibration band at 1376 cm^{-1} assigned to bending of the C-H group in cellulose of the substrate, also decreased with the enzymatic treatment when compared to untreated substrate. This decrease was highest in case of WH which correlated with the results of the saccharification where highest sugar release was observed in case of WH substrate. Peaks at 1429 and 893 cm^{-1} were attributed to the cellulose with crystalline and amorphous nature of cellulase which was persistent after enzymatic saccharification indicating that the biomass substrates might require further physicochemical treatment to alter the crystallinity of cellulose as suggested by Kristensen et al. (2008). Absorption bands at 1251, 1046 and 990-996 cm^{-1} were attributed to hemicellulose components. And the absorption peaks observed at these wavenumbers also supported the results as the intensity of peak decreased with the treatment of enzyme having enhanced components. The complex structure of lignin displayed numerous vibration bands as observed at 1127, 1508, 1597 and 1654 cm^{-1}

due to variable to medium C-C stretching for aromatic ring (Rajak and Banerjee, 2015, 2016). Similar absorption band patterns as mentioned earlier have been reported in literature for recovered cellulase, hemicellulose and lignin rich samples (Kuila et al., 2011; Magalhães da Silva et al., 2013)

Thus, when different cocktail formulations of CP, CX, PX, and CPX applied on raw untreated agrowaste biomass of BH, SCB and WH, positive fold increase in saccharification was observed which proved the synergism of xylanases and pectinases with each-other and with commercial cellulase. This has also confirmed their potential accessory role in cocktail used for saccharification studies. The morphological and structural analysis based on SEM and FTIR analyses supported the saccharification studies, where the untreated biomass showed intact surfaces while the cellulase and CPX cocktail hydrolysed biomass gave a flaky appearance due to enzymatic destruction of the cell wall.

As per literature survey, the SCB biomass is more used for cogeneration of heat and energy in India. Significant efforts are being done to increase its amenability for saccharification and alcohol production and yet the full potential is not achieved. Based on the above observations, sugarcane bagasse (SCB) biomass was further selected for improvement of saccharification which included physicochemical pretreatment and enzymatic saccharification.