Conclusion

In the present era of green energy, production of bioethanol from biomass is the leading process which involves two steps in a sequence as saccharification and fermentation. Saccharification, digestion of plant biomass structural polysaccharides to its constitutive monomer units, is a keystone step in the biofuel production as it controls the availability of the fermentable sugar for the subsequent fermentation step. Enzymatic saccharification of such lignocellulose biomass is more beneficial than the chemical saccharification as the harmful end-products are negligible. But, the complex nature of lignocellulosic matrix in cell wall, along with crystalline and amorphous nature of cellulose creates a huge hindrance and restricts the rate of enzymatic saccharification process. Removal or relocation of lignin from the complex matrix can be achieved by either chemical pretreatment or by enzymatic action while the hemicellulosic hindrance of xylan, pectin, mannan etc., can be overcome with help of accessory enzymes like xylanase, pectinase, mannanase etc., which hydrolyze them and expose the cellulose fibers for improved saccharification by enhancing their accessibility to the core group of cellulase enzymes. Thus, core and accessory enzyme cocktail mediated saccharification of pretreated biomass can result in the improved yield of fermentable sugars.

As India cultivates the second highest amount of sugarcane after Brazil, sugarcane bagasse (SCB) is highly available as cost effective by-product from sugar mill factories. SCB due to its higher cellulose and hemicellulose content is a suitable substrate for conversion to sugars which is required for biofuel production. Literature regarding the saccharification process, diverse pretreatments and combinations of plant polysaccharide degrading enzymes is vast and still the maximum saccharification to its full potential is not yet realized by any system reported uptill now.

In this perspective, bacteria that can produce plant cell wall degrading enzymes were isolated from the different niches comprising digested or decayed plant materials. Crude xylanase and pectinase from these isolates were further employed in the saccharification studies of raw agrowaste biomass to study synergism between them. Besides this, several pretreatments were applied for biomass deconstruction to improve its accessibility to plant cell wall degrading bacterial enzymes and further different cocktails of accessory xylanase and/or pectinase with commercial core cellulase for saccharification were studied. The physicochemical characterization of xylanase/xylan hydrolase was performed to further understand its suitability for saccharification

process. At the end, as these xylanases and pectinases both served as accessory enzymes, the concurrent production of xylanase and pectinase was optimized. The major conclusions drawn from the findings presented in the thesis are noted as below.

Primary qualitative screening concluded that, the selected sources for isolation were predictably dominated by the bacteria that could produce cellulase, xylanase and pectinase all three enzymes following by the cellulase-xylanase producers and further pectinase-xylanase and cellulase-pectinase producers. Ruminant dung seemed to be a suitable source for the producers of these enzymes. Keeping in mind the application of enzymes in saccharification step of biofuel ethanol production, the plate assays were carried out with crude substrates like CP, WB and FP in addition to pure ones. Further, quantitative assays, microscopic and morphological data yielded 54 promising isolates that could degrade the crude plant derived substrates out of which seven were able to produce both xylanase and pectinase enzymes. Phylogenetic analysis based on ARDRA did not sort the 54-high xylanase, pectinase and both enzyme producing isolates as it grouped 51 isolates in a single cluster with *Bacillus pumilus* group except the three. Therefore, the seven bacterial isolates producing cellulase free xylanase and pectinase together were chosen and identified as *B. safensis* or *B. altitudinis* on the basis of 16S rRNA and *gyrB* gene sequence analysis. 16S rRNA gene sequencing was aided by *gyr*B gene sequencing and biochemical tests to get accurate identification. Higher activities of the crude enzymes at 40-60 °C and in pH 6.0-9.0 range, the attributes necessary for saccharification process, narrowed down the selection to final three cultures viz. *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 for further application-based studies. From the literature, xylano-pectinolytic *Bacillus* species are predominant and widely exoploited for varied applications. They belong to biosafety level-1, can be cultivated on simple media, are easy to maintain and digest common agrowastes, hence were organism of choice for the studies undertaken here.

Cocktails formulated from crude xylanase (X) and pectinase (P) of these three *Bacillus* cultures with each other or with the commercial cellulase (C) viz. PX, CP, CX, and CPX in this order produced increase in % saccharification on raw agrowastes barley husk (BH), sugarcane bagasse (SCB) and wheat husk(WH). These enzymes cocktails were effective on different agrowaste substrates of varying composition. Positive synergism was observed among the cocktails PX, CP, CX, and CPX as the practical yield of saccharification exceeded the cumulative yield of components present in case of each cocktail. SEM and FTIR analyses concluded that the supplementation of these

crude xylanase and pectinase in commercial cocktail enhanced the structural degradation of raw biomass and the diminution of cellulosic and hemicellulosic content when compared with only cellulase treated biomass. This concluded the accessory role of crude xylanase and pectinase enzymes to the core commercial cellulase since they enhanced cellulase saccharification activity. However, the low yield by CPX cocktail during saccharification of raw biomass (10.58% with BH, 9.68 % with SCB and 16.14 % with WH) suggested that the cellulose accessibility was limited possibly due to the hindrance caused by lignin components and the complex matrix created by lignin with hemicellulose and cellulose components. The industrial importance, bulk biomass availability and low cost makes SCB desirable substrate for saccharification and therefore was selected for further studies.

Screening of pretreatment methods comprising hydrothermal pretreatments like autoclave and steam explosion as well as chemical pretreatments like NaOH, NH_4OH and H_2SO_4 was performed to decide a suitable pretreatment method for SCB. Amongst these, highest reducing sugars released from SCB was observed in case of a H_2SO_4 pretreatment filtrate. The HPLC analysis of this filtrate suggested that the majority of sugar released was xylose, thereby implying that the acid treatment removes the hemicellulosic fraction from biomass keeping cellulose fractions unharmed. NaOH and NH_4OH treatment released negliegible amount of sugars in the filtrate but HPLC unveiled release of soluble components other than sugars in these two cases. Hydrothermal pretreatments removed neither sugars nor other solubles. Gravimetric analysis also supported these observations as it was found that NaOH and H_2SO_4 pretreatments exhibited higher loss in dry weight of biomass than other pretreatments. To sum up, the biomass weight loss, reducing sugars released and other solubles released were the parameters leading to a conclusion that NaOH treatment was most suitable for SCB biomass. Further support to this observation was obtained from FTIR and SEM analysis, which also indicated that highest lignin loss was observed in NaOH pretreatment, which disrupted the structure of SCB biomass by releasing the parenchymatous pith cells from vascular bundles giving it more fibrous appearance. Commercial cellulase gave 2.58% saccharification from raw SCB biomass which enhanced to 38.8% in case of NaOH pretreated SCB. Increase in saccharification by individual xylanases and pectinases from this study too, was appreciable and comparable to reported crude enzyme saccharification values (details in Table 4.4)**.** Increase in biomass saccharification after NaOH and other pretreatment methods

implied that there was improved accessibility of cellulosic and hemicellulosic polysaccharides from SCB biomass to their respective enzymes, i.e. cellulase, and xylanase and pectinase. Even after pretreatment, only ~38.81% of biomass saccharification by commercial cellulase suggested that the process still can be improved and further studies with application of different combinations of enzyme cocktails were pursued.

For saccharification studies of pretreated SCB (PSCB) biomass, further cocktails of the crude xylanase and/or pectinase along with commercial core cellulase were formulated by using either additive approach or substitutive approach. In the former, crude accessory enzyme from individual isolates were used to form cocktails while in the latter crude xylanase and pectinase enzymes from all three isolates were substituted in commercial cellulase mediated cocktail. Comparison of biomass saccharification yield for both the approaches revealed that the substitutive cocktails in each case, i.e., PX, CX, CP and CPX exhibited enhanced saccharification of biomass. Thus, when additive and substitutive cocktails were compared for the saccharification yield, the substitutive cocktails were found more effective over additive cocktails as all substitutive cocktails enhanced the saccharification yield. From NaOH PSCB, substituted CPX cocktail gave highest saccharification of 84.02% than the additive CPX cocktails (81.30% by M35-CPX, 72.24% by R31-CPX and 71.4-81.3% by J208-CPX). The reason for more sugar yield by the substitutive cocktail can be attributed to the variety of activities present in it. These activities could be contributed by the crude xylanases and pectinases of the selected bacterial cultures that are complementary to each other. The maximum yield of 84.02%, obtained in the present studies, was comparable to the reported saccharification values of 80-85% by optmimized cocktails of commercial enzymes like, Celluclast 1.5L, Spexyme CP, Multifect Xylanase and Multifect Pectinase (details in Table 5.7).

Also, substitutive xylanase cocktails prepared from three enzymes M35, R31 and J208 gave enhanced saccharification than individual xylanase enzymes demonstrating compatibility of all the three xylanases with each other. Similarly, the three pectinases together gave enhanced saccharification than individual enzymes in substitutive cocktails. This also indicated that even though the individual M35, R31 and J208 enzymes possessed similar physicochemical characteristics the enzyme activities present in the crude CFS varied. The crude enzymes might be having activities on different bonds of the polysaccharide backbone due to which the polysaccharide was

more simplified easing the saccharification process. Thus, the three xylanases and pectinases were on par with each other and formed a blend that could expand the conditions in which they act.

Hydrolase activity was detected in the crude xylanases, while polygalacturonate hydrolase, pectin lyase and pectate lyase activities were detected in the crude pectinase. Predictably these were the activities responsible for saccharification. The crude xylanases and pectinases were found to be cellulase free, exhibited broad specificity and affinity for polysaccharide substrates and performed the role of accessory enzymes to the core commercial cellulase preparation for saccharification.

As the noteworthy accessory role of xylan hydrolase activity from each isolate was concluded from saccharification by CX, and CPX cocktails it was characterized for physicochemical properties. These M35, R31 and J208 crude xylanases were mesophilic in nature with optimum activities at 50 °C and stable in range of temperature 40-50 °C. The optimum pH observed for all three xylanases were in range of pH 7.0 to 8.0 and they were stable between range of pH 6.0 to 9.0. The pH range of the xylanases will be expanded from acidic to alkaline condition (pH 5.0-9.0) at 40-60 °C temperature when present in cocktail, which is favourable for saccharification of biomass. The temperature of 40 °C can be recommended for applications involving long-term incubation from the results obtained. The drastic decrease in viscosity and absence of reducing sugar monomer end-products indicated the endo acting nature of xylanase. This was also proven by TLC and HPLC analysis. Endo-acting enzymes are preferred as accessory enzymes since they contribute majorly to the deconstruction of the polysaccharide on which they act. Application of modulators like Cu^{2+}, Ca^{2+} and 2-mercaptoethanol increased the enzyme activities suggesting that the activity can further be improved. Thus, these physicochemical conditions can be further applied as incubation parameters for saccharification process where they will have cumulative effect of enhancing the saccharification process.

Further the potentials of crude xylanase and pectinase enzymes for their application as accessory enzymes in industrial application led to studies on optimization of inducer substrates in media which can induce the concurrent production of both xylanase and pectinase enzymes in single medium. The optimization helped to arrive at an optimum medium amended with WB and CP as suitable inducer substrates giving enhanced concurrent production of xylanase and pectinase. Notably WB and CP induced both the enzymes in the culture used here.

To sum up the entire studies M35 CPX cocktail gave 8.13% saccharification on raw SCB, which increased to 81.30% in case of NaOH treated SCB in additive cocktail. In substitutive cocktail where xylanases and pectinases from all the three *Bacillus* culture were present, this value reached 84.02%. The cocktail bears potential of carrying it further for scale-up studies.

Thus, in conclusion from the findings of the present investigations, the crude xylanases and pectinases from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 when applied with commercial cellulase Primafast®200, exhibited a clear synergism in saccharification of BH, SCB and WH. The immense improvement in performance of the cocktail after supplementation of xylanase and pectinase enzymes to commercial cellulase suggests their great potential to act as accessory enzymes when supplemented to core commercial cellulase. The involvement of pretreatment like NaOH also significantly enhanced the saccharification process of SCB biomass. The optimized concurrent production of these accessory enzymes from the selected *Bacillus* cultures on cost effective substrates can further help in the development of economically viable process. The defined enzyme cocktails prepared for specific agrowaste like in this case SCB would provide optimal saccharification from it. Hence the scale-up of these studies at industrial plant level with the cocktails prepared in the investigations carried out in this work will give better understanding about their performance at upgraded level. The future of an economically feasible enzymatic hydrolysis process is reliant on reduced production costs and an increase in specific activities for enzymatic mixtures. Hence, further combination of accessory enzymes concurrently produced on optimized media with different available cellulases can provide tailor-made enzyme cocktails which will efficiently augment the saccharification process of different agrowaste biomass. Besides their application in saccharification, these crude xylanases and pectinases, due to their cellulase free nature, can be explored for their application in pulp and paper industries, textile industries, food industries.