Abstract

In biofuel production from agrowaste, the first step of saccharification is the keystone step since it provides the sugars for subsequent ethanol production. Agrowaste saccharification is carried out by core cellulases and presence of lignin, complex hemicelluloses and pectin in the cell wall matrix of plant derived agrowaste biomass decrease their potential activity by restricting the cellulose accessibility. Such limitations necessitate the requirement of pretreatment for removal or relocation of lignin and a cooperation from different accessory enzymes such as xylanases and pectinases which enhances the availability of cellulose to cellulase for biomass saccharification by removing the hemicellulose and pectin covering.

Screening based on qualitative plate tests, quantitative assays, microscopic and morphological data and ARDRA analysis of 468 bacterial isolates for polysaccharide degrading enzymes from niches like ruminant dung, yard manure, rotten wood etc., yielded seven promising isolates. Using 16S rRNA and *gyrB* gene sequence analysis, these seven cellulase free xylanase and pectinase producing isolates were identified as *Bacillus safensis* M18, M33 and M35 as well as *Bacillus altitudinis*, R30, R31, J208 and J216. *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were selected for further studies.

Crude xylanase (X) and pectinase (P) obtained from the above isolates and commercial cellulase Primafast®200 (C), either individually or as formulated cocktails viz., PX, CX, CP and CPX were used for saccharification of raw agrowastes like Barley husk (BH), Sugarcane bagasse (SCB) and Wheat husk (WH). Synergism was observed between PX, CX and CP as well as among CPX confirming the accessory role of xylanases and pectinases in the core cellulase containing cocktail. CPX cocktails gave highest 7.8-9.7% saccharification on raw SCB biomass. SCB biomass was subjected to pretreatment agents like steam under pressure (autoclave and steam explosion), NaOH, NH_4OH and H_2SO_4. Scanning electron microscopic (SEM) analysis of different pretreated biomass exhibited structural changes in the pith cells of biomass and fibrous appearance particularly in NaOH pretreatment. FTIR spectra of NaOH and NH_4OH pretreated biomass exhibited reduction in lignin suggesting its removal by these pretreatments, while reduction of hemicellulose was observed in H_2SO_4 pretreatment. HPLC analysis of the pretreatment filtrates also supported these observations. Fluorescent microscopy of cell wall and sugar estimation from enzyme hydrolysate further established the enhanced accessibility of cell wall polysaccharides of pretreated

SCB (PSCB) to their enzymes. Each pretreatment enhanced the enzyme cocktail mediated saccharification of PSCB than raw SCB but the highest increase was observed in case of NaOH pretreatment. Commercial cellulase alone exhibited maximum of 2.20% saccharification from raw SCB which was further enhanced to 38.8% in case of NaOH PSCB.

Additive CPX cocktails exhibited 71.42-81.30% saccharification of NaOH pretreated biomass while substitutive CPX cocktail enhanced biomass saccharification to 84.02% suggesting substitutive cocktails were more efficient in saccharification over additive cocktails. SEM images exhibited disrupted and flaky appearance of PSCB, which were more pronounced after enzymatic saccharification when compared with raw unhydrolyzed untreated biomass. Reduction of cellulose and hemicellulose fractions in FTIR spectrum also supported the observations.

Characterisation of M35, R31 and J208 xylanases demonstrated high specificity and affinity for birchwood xylan over beechwood xylan. They were found to be mesophilic (40-55 °C) in nature, stable and active in pH 6.0-9.0. Viscosity and end-product analysis by HPLC revealed their endo-acting nature. Certain common modulators like, Ca^{2+}, Cu^{2+} and 2-mercaptoethanol were found to serve as enzyme activators for these xylanases. CP and WB were identified as potential inducer substrates for production of xylanases and pectinases. Their concentrations were optimized using quadratic model in central composite design (CCD) of response surface method (RSM). *B. safensis* M35 and *B. altitudinis* R31 and *B. altitudinis* J208 produced maximum of 14.9-18.2 xylanase units. *B. safensis* M35 produced 695.5 pectinase units whereas *B. altitudinis* R31 and J208 produced ~1465 pectinase units.

In the present study positive synergism among the xylanases and pectinases from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 as well as with commercial cellulase was clearly demonstrated. Their role as accessory enzymes in commercial core cellulase mediated saccharification of agrowaste biomass was thus established. Xylan hydrolase and Pectin lyase were the major activities present in the crude enzymes produced by three *Bacillus* strains. Physicochemical characterization of xylan hydrolases prooved their suitability for their application in saccharification process of bioethanol production. Concurrent production of xylanase and pectinase using optimized crude polysaccharide agrowaste inducer substrates will help in production cost reduction. Further scale up studies of this saccharification system are warranted to assess its potential.