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Pilot scale pretreatment of wheat straw and comparative evaluation of commercial enzyme preparations for biomass saccharification and fermentation

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ABSTRACT

Conversion of pretreated lignocellulosic biomass (LCB) into sugars is one of the critical steps for bioethanol production. High LCB hydrolysis could be achieved by employing robust enzymes having high inhibitor tolerance, low irreversible lignin binding, and low end-product inhibition. In this study, acid pretreatment of wheat straw was carried out at pilot scale (250 kg/day) and three commercial cellulase preparations from Advanced Enzyme (AD), Novozyme (CL), and Genencor (AC) were evaluated for inhibitor (lignin, furfural, hydroxyl methyl furfural, vanillin) tolerance. Pretreated wheat straw (PWS) hydrolysis was carried out at different enzyme concentrations (1–30 mg protein/g of PWS) under optimum pH and temperature in rolling bottle reactor. Simultaneous saccharification and fermentation was performed employing in-house thermotolerant *Saccharomyces cerevisiae*. Results indicated that, maximum saccharification (more than 85%) was achieved at low protein loadings (10–15 mg protein/g PWS) of CL and this enzyme was also found to be more robust in presence of inhibitors. Maximum ethanol yield (78%) was found at 20 mg protein/g of PWS using CL. This study suggests that inhibitors have significant detrimental effect on enzymes and better understanding of enzyme-inhibitor correlation with its critical moderation would help in further enhancing the LCB hydrolysis at low enzyme dosage.

1. Introduction

Bioethanol from lignocellulosic biomass (LCB) is considered as a potential alternative to transportation gasoline. It will help in reducing the environmental pollution and also provide energy security for a developing country like India. The process involves three critical steps; first is pretreatment, which is a thermo-chemical process, carried out at high temperature and pressure conditions. This helps in the development of pores in LCB matrix so that; enzymatic hydrolysis (second step) of LCB in to fermentable sugars can take place. In third step, ethanol is produced by fermentation of sugars by using ethanologic yeast.

Pretreatment is carried out to reduce the recalcitrance of the LCB. Most of the prevalent pretreatments (especially dilute acid pretreatment) are performed under severe conditions, resulting in formation of phenolic and furfural compounds from degradation of lignin and sugars [1–3]. These compounds include simple phenolics (vanillin, ferulic acid, syringaldehyde, conifer alcohol, etc.), oligomeric phenolics (ellagic acid, epicatechin, tannic acid, etc.), furfural (hydroxyl methyl furfural and furfural), and acids (acetic, formic, and levullinic acids). The amount and type of phenolic compounds depend on the type of LCB, pretreatment methods and conditions [1]. The inhibitory effect of these compounds on fermentation is widely reported however, very few reports are available regarding their effect on enzymes and LCB hydrolysis [1,4].

One of the major challenges for the commercialization of second generation ethanol technology is the high cost of enzymatic depolymerization of pretreated LCB [5–7]. This may be attributed partially to the costly cellulase available in the market and high concentration of cellulases required to overcome the inhibitory/inactivating effects of lignin, phenolics, furfurals, and acids. The lignin present in LCB acts as a barrier to prevent the access of cellulolytic enzymes to the substrate. It also binds unproductively with cellulase and decrease cellulase availability for saccharification. A significant reduction in saccharification cost may be achieved if low concen-

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tration of enzyme is used to achieve complete hydrolysis in short time.

Commercially, filamentous fungi mainly *Trichoderma* and *Aspergillus* species are the preferred enzyme producers. These fungi produce a spectrum of enzymes including cellulase endoglucanases (1,4-β-D-glucan glucanohydrolases), exoglucanases (1,4-β-D-glucan cellobiohydrolases), hemicellulase complex (endo-1,4-β-D-xylanases, 1,4-β-D-xylosidases, endo-1,4-β-D-mannanases, 1,4-β-D-mannosidases, α-D-galactosidases, α-l-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases, and feruloyl and p-cumaric acid esterases) and other accessory enzymes, such as glycoside hydrolase belonging to family 9, 48, and 61 which are essential for LCB hydrolysis [8]. These enzymes work synergistically to cleave cellulose and hemicelluloses bonds effectively. Currently, enzyme industries, such as Novozymes, Denmark (Cellic CTec 2 and 3), DuPont–Genencor, USA (Accelerase 1500, XP, XC, BG, and Duet), Dyadic International, USA (AlternaFuel CMAX, Dyadic CMAX) are manufacturing multi-enzyme preparations for producing fermentable sugars in an efficient manner. Some of these advanced enzyme preparations contain high β-glucosidase and lytic polysaccharide monooxygenases (LPMO) activity which make them less susceptible for end product inhibition and improved the overall performance of the enzymatic cocktail [9]. The cost of cellulase preparations from Genencor, Novozymes, and other manufacturers has been reduced significantly (20-fold) over the past decade [10]. Recent state of technology cost estimates indicate that enzymes remain the significant contributor to operating cost in the cellulosic ethanol process, after feedstock, representing an estimated cost of approximately US $0.30–US $0.50 per gallon of ethanol [11]. Thus, LCB saccharification still remains a key cost barrier and further reduction in enzyme cost is needed [12]. Indeed, limiting cellulase inactivation is a strategic alternative to the development of efficient enzyme cocktails at affordable price [13].

Fermentation is the final step to produce ethanol from fermentable sugars. Two approaches are predominant for ethanol production. If fermentation is carried out after completion of enzymatic hydrolysis of pretreated LCB, it is known as separate hydrolysis and fermentation (SHF). In another approach enzymatic hydrolysis and fermentation are carried out in a single step this is known as simultaneous saccharification and fermentation (SSF) [14]. In SSF, the released sugars from enzymatic hydrolysis are simultaneously consumed by the fermenting microorganism, for example, *Saccharomyces cerevisiae* during fermentation avoiding product inhibition of enzymes and also decreasing the probability of contamination. Reduced number of process reactors is one of the features of SSF, which integrates enzymatic hydrolysis and fermentation in one reactor [15,16]. However, enzyme's optimum temperature (50–55 °C) needs to be compromised for ethanol production by yeast or a thermotolerant yeast needs to be used.

In this study, wheat straw was pretreated in a continuous pilot scale pretreatment plant with dilute acid. Composition analysis of untreated and pretreated wheat straw was carried out and three commercial enzymes from different enzyme manufacturing majors were evaluated for enzymatic hydrolysis and SSF process using rolling bottle reactors. The inhibitory effects of model compounds (lignin, acetic acid, furfural, hydroxyl methyl furfural, and vanillin) generated during dilute acid pretreatment were studied for pure cellulose (avicel) hydrolysis using three commercial enzymes. This allowed estimating the inhibitory effects of inhibitors on different commercial preparations. The concentrations of these inhibitors were analyzed in the hydrolysate and similar concentrations were used to evaluate their dynamics during the fermentation process and to test their inhibitory effects individually using a screening method [17].

2. Material and methods

2.1. Materials

All chemicals (analytical grade), such as sulfuric acid, sodium hydroxide, cellobiose, glucose, xylose, alkali lignin (AL), furfural, hydroxyl methyl furfural (HMF), BCA-1 kit, and acetic acid used for quantification of sugars were procured from M/s Sigma–Aldrich, India. Wheat straw (WS) was procured from local market in Faridabad, Haryana, India. It was grounded by using high speed cutting mill (Texol, Pune, India) in to 1–2 mm size. Three commercial enzyme preparations Sacchari-SEB C6 (Advanced Enzymes), Mumbai, India (AD), Novozymes, USA (CL), and Genencor, Denmark (AC) were obtained from different vendors.

2.2. Pilot scale dilute acid (DA) pretreatment

Pretreatment of WS was carried out in 250 kg/day continuous pilot-scale pretreatment reactor system using dilute sulfuric acid. The pretreatment system included a size reduction mill, high temperature and pressure reactor, flash tank, hydraulic press, and a weight loss type feed hopper. The milled WS was presoaked in the acid solution for 30 min followed by pressing in a hydraulic press to remove excess liquid. The WS was fed to the feed hopper, which maintains the desired feed rate of 10 kg/h. Material exits through a conveyor belt that delivers it to a plug mill that compresses the material into a strong solid plug that is then pushed into the pretreatment reactor. This unique arrangement helps to maintain the steam pressure in the reactor while continually injecting the feed into the reactor. After passing through the screw type pretreatment reactor, the pretreated material reaches to a flash tank. Pretreatment was carried out under previously optimized conditions (data not shown here) i.e., at 160 °C temperature, 5.2 bar pressure, 10 min residence period and 0.5% (v/v) sulfuric acid. The pretreated wheat straw (PWS) slurry (containing cellulose, hemicelluloses, and lignin) was collected in the slurry tank. This was transferred through a pump to a high speed centrifuge for separating solids (mainly cellulose and lignin) and liquid (mainly pentoses) [18].

2.3. Compositional analysis of pretreated wheat straw

Compositional analysis was carried out by following the Laboratory Analytical Procedure (LAP) of National Renewable Energy Laboratory (NREL) [19] to determine the glucan, xylan, lignin, ash, and extractives content in pretreated or untreated wheat straw. Various sugars (glucose, xylose, arabinose etc.) and inhibitors (furfural, HMF, acetic acid etc.) found in pretreatment slurry, were analyzed by high performance liquid chromatography (HPLC; Waters, Germany) equipped with a BioRad Aminex HPX-87H column (BioRad Hercules, CA) at 50 °C, 0.008 N H_2SO_4 at a flow rate of 0.6 mL/min as mobile phase. Sugars were detected on a refractive index detector (RID) while inhibitors were detected on UV-detector. All analyses were conducted in triplicate.

2.4. Enzyme assays

β-Glucosidase activity was determined as described previously by Agrawal et al. [6]. One unit (U) of β-glucosidase was defined as the amount of the enzyme which would produce 1 μmol *p*-nitrophenol per min under the standard assay conditions and the specific activity was defined as the number of units per milligram (mg) of protein. Filter paper units (FPU) and endoglucanase (CMCase) activity were analyzed according to the method