Journal of Bioprocessing & Biotechniques
ISSN: 2155-9821
Choudhary et al., J Bioproces Biotech 2014, 4:7
DOI: 10.4172/2155-9821.1000188

Research Article Open Access

Enhanced Saccharification of Steam-Pretreated Rice Straw by Commercial Cellulases Supplemented with Xylanase

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Abstract

For successful commercialization of biomass to ethanol process, the techno- economic hurdles have to be overcome. Complete substrate utilization is the key for making economics favorable. Existing enzyme preparations do not give high saccharification efficiency, thus, a broader suite of hydrolases is required. Xylanase supplementation in enzyme cocktail is an important strategy to increase sugar yields. In our study, hyperxylanolytic *Aspergillus awamori* F18 was used to produce xylanase. *A. awamori* F18 expressed high levels of xylanase during Solid state fermentation of corncob and also, Carboxy Methyl Cellulase (CMCase), filter paper activity (FP lyase) and β-glucosidase (524.43, 43.95, 8.64 and 29.81 IU mg^{-1} proteins respectively). A 10.6-12.5 fold concentration of these activities was achieved by single-step acetone precipitation. Supplementing concentrated xylanase to Accellerase®1500 resulted in 69.5% enhancement in sugars released after 72 h of saccharification of steam-pretreated rice straw. HPLC analysis of hydrolysates showed higher glucose levels along with the presence of xylose and arabinose. There was a 12.4% increase in the amounts of glucose as well as total sugars released when xylanase was supplemented to commercial cellulase and β-glucosidase.

Keywords: Bioethanol; Lignocellulosic biomass; Enzymatic saccharification; Xylanase; Cellulase

Introduction

Depleting fossil fuel reserves, dwindling fuel prices, increased concerns over climate change, and most importantly the search for energy security have led the global research community to look for renewable, environment friendly and sustainable energy sources. In this perspective, ethanol derived from biomass can be used to supplement conventional fossil fuels and cut down on petroleum consumption. For first generation bioethanol, industry mostly depended on sugar and starchy feed stocks. Thus, this option is limited to the countries where actual and sustainable surplus of these crops is available. Moreover, since these feed stocks are a part of food, this resulted in a sudden increase in food prices all over the globe. This led to the concept of 2^{nd} generation bioethanol, which is derived from lignocellulosic biomass and do not threaten food security. In developing countries like India, the possible competition with food is one of the risky factors when using agricultural crops for ethanol production. In this regard, lignocellulosic biomass, which is not a part of human food chain, is envisaged to supply a significant portion of the feed-stocks for bioethanol production in the medium and long term due to their low cost and abundance. Lignocellulosic biomass constitutes cellulose and hemicellulose (carbohydrate polymers), and lignin. Cellulose is a linear, crystalline homopolymer made up of repeated units of glucose strung together with β-glycosidic bonds whereas hemicellulose is a highly branched heteropolymer consisting of xylose, glucose, galactose, mannose, and arabinose. The exact sugar composition of hemicellulose varies depending on the plant type and origin.

Biomass conversion to ethanol proceeds in a step wise manner where pretreatment and saccharification are the two most important steps which determine the overall cost and efficiency. Saccharification can be carried out by chemical or biological means by employing glycosyl hydrolases. Bacteria and fungi are good sources of cellulases and xylanases that cause the breakdown of cellulose and hemicellulose chains in order to produce monosaccharides for the subsequent fermentation step. Hydrolysis of cellulose is brought about by cellulase complex while hydrolysis of hemicelluloses involves enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endo-hemicellulases and others, the concerted action of which hydrolyse glycosidic bonds, ester bonds and remove the chain's substituents or side chains [1]. These include endo-1, 4-β-xylanase, β-xylosidase, β-mannanase, β-mannosidase, α-glucuronidase, α- L-arabinofuranosidase, acetyl xylan esterase and other enzymes. Although the enzymatic saccharification is inherently slow process, it demonstrates better results using pretreated lignocellulosic biomass because no degradation products are formed which inhibit fermentation step. The other prominent advantages of enzymatic hydrolysis are the mild process conditions which do not require expensive materials of construction and low process energy requirements. Thus, enzymatic hydrolysis of biomass carbohydrates is the technology of choice. However, existing technologies and available cellulase cocktails do not allow complete saccharification of plant polymers as there are many factors hindering the action of cellulases. Currently employed cellulases are sourced from saprophytic fungi [2] and these cellulase preparations are subject to tight induction and regulation systems and also inhibition from various end products [3,4]. Therefore, more potent and efficient enzyme preparations need to be developed for the enzymatic saccharification process to be more economical. To overcome the limitation of enzymes and to get higher saccharification efficiency, a broader suite of enzymes is required for hydrolysis of cellulose and hemicelluloses to fermentable sugars [2,5]. Additional critical enzymes need to be present in commercial cocktails at optimal levels for improving the efficiency of these enzyme mixtures [6]. Also, the xylan and xylan fragments present in the biomass have been suggested to be one of the causes of the

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Received October 16, 2014; **Accepted** November 26, 2014; **Published** December 01, 2014

Citation: Choudhary J, Saritha M, Nain L, Arora A (2014) Enhanced Saccharification of Steam-Pretreated Rice Straw by Commercial Cellulases Supplemented with Xylanase. J Bioprocess Biotech 4: 188 doi: 10.4172/2155-9821.1000188

reduced activity of cellobiohydrolases [7]. Therefore, supplementation of enzyme cocktails with xylanases can alleviate the problem of inhibition of cellulase enzyme action caused by xylan oligosaccharides. Xylanase supplementation in combination with other pretreatments and cellulase improves sugar yields [8,9]. The reactivity of component enzymes differs with the enzymatic activity and biomass, and for higher saccharification ability, cellulases need high hemicellulase activity as well as high β-glucosidase activity [10]. However, there are very few reports on supplementation of cellulase cocktails with xylanases for increasing saccharification yields. The present study was conducted to produce xylanase from a hyperxylanolytic fungal strain, *Aspergillus awamori* F18 and assess the impact of xylanase supplementation to standard cellulases (Accellerase®1500, commercial cellulase and β-glucosidase) on enhancement of sugar yields during saccharification.

Materials and Methods

Organism

The organism used in the study was *Aspergillus awamori* F18 obtained from the culture collection, Division of Microbiology, Indian Agricultural Research Institute, New Delhi. The fungus was grown on Potato Dextrose Agar (PDA) medium at 30°C. After one week of incubation, the slant cultures were stored in refrigerator at 8°C until used.

Qualitative assays of xylanolytic and cellulolytic abilities

Xylanolytic potential of *A. awamori* F18 was evaluated on the basis of its ability to grow on Reese's mineral medium with 0.1% xylan as sole carbon source. The ability of *A. awamori* F18 to produce endoglucanases was assessed by its ability to produce zones of hydrolysis on CMC (Carboxy Methyl Cellulose) agar (Congo-red test), as described earlier [11].

Xylanase production using A. awamori F18

Xylanase was produced by *A. awamori* F18 under both submerged and solid state fermentation by using different substrates. For submerged fermentation, 0.1% xylan or 1% rice straw was used as substrate and for solid state fermentation, corn cob was used.

Production of xylanase by *A. awamori* F18 under submerged fermentation

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml Reese's mineral medium supplemented with 1% rice straw / 0.1% xylan as carbon source. The flasks were autoclaved at 15 psi pressure and 121°C temperature for three consecutive days. They were inoculated with three agar plugs (6 mm) of fungal mycelia and incubated at 28°C for 7 days in stationary condition. After incubation, the contents of the flasks were filtered through Whatman No. 42 filter paper and the filtrate was used for estimation of different enzyme activities after 3, 5 and 7 days of incubation.

Production of xylanase by *A. awamori* F18 under solid state fermentation

Solid state fermentation was carried out using corn cob as substrate. The substrate was dried and ground to pass through a 2 mm sieve. Erlenmeyer flasks (500 ml) containing 5 g substrate with 25 ml Reese's mineral medium was autoclaved at 121°C temperature and 15 psi pressure for three consecutive days and inoculated with 1 ml spore suspension (4×10^6 spore/ml) of *A. awamori* F18. Flasks were incubated at a temperature of 30°C for 7 days. Treatments were carried out in triplicates. After 7 days of incubation, 70 ml of sodium citrate buffer (0.05 M, pH 4.8) was added to each flask and contents were mixed on a shaker at 200 rpm for 2 hours for extraction and vacuum filtered. The filtrate was used as source of enzymes and assayed for xylanase, cellulases and proteins.

Enzyme was also produced on large scale by solid state fermentation of 100 g of corncob in glass trays. All the parameters regarding inoculation, incubation and extraction were kept the same.

Xylanase and cellulase activity assays

Xylanase activity was assayed by the method described by Ghose and Bisaria [12]; filter paper (exo-β-glucanase) activity and CMCase activity (endo-β-1, 4-glucanase) were assayed as described by Ghose [13]. The reducing sugars released were measured by the DNSA method [14]. One International Unit of xylanase was expressed as 1 µmole of xylose formed per minute and one International Unit of filter paper activity or CMCase corresponded to 1 µmole of glucose formed per minute during hydrolysis. β-glucosidase assay was performed using p-nitrophenyl-β-D-glucopyranoside as substrate [15] and the activity was calculated in terms of µmoles of p-nitrophenol produced per ml of culture filtrate per minute.

Concentration of enzyme

Crude enzyme extract produced by solid state fermentation was concentrated by acetone precipitation. The enzyme extract was centrifuged at 8000 rpm for 10 min to remove the impurities present in crude extract. This was then mixed with chilled acetone (stored at -20°C) in a ratio of 1:5 and kept at 4°C overnight. The protein precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. Pellet was air dried and dissolved in sodium citrate buffer of pH 4.8. Concentrated xylanase enzyme was used for enzymatic saccharification of pretreated rice straw.

Biochemical characterization of enzyme

Temperature and pH optima of enzyme activities were determined. To study the effect of temperature on the activity, reaction mixture was incubated at different temperatures i.e.35, 45, 50, 55, 65 and 75°C. Enzyme assays were carried out as described earlier. To determine optimum pH for xylanase, cellulase, FPase and β- glucosidase activities, respective substrates were dissolved in 0.05 M sodium citrate buffer of pH ranging from 3-6, Sorensen phosphate buffer of pH 7-8 and glycine NaOH buffer of pH 9. The respective buffers were used for enzyme assays at the particular pH. Activities were measured after incubation with enzyme.

Saccharification of rice straw with commercial cellulases supplemented with xylanase

To assess the effect of xylanase supplementation on hydrolysis of rice straw and sugar yields, the concentrated xylanase preparation from *A. awamori* in combination with Accelerase®1500 (Genencore) or cellulase (Sigma) + 0.1 ml β glucosidase (Sigma) were used.

Pretreatment of rice straw for saccharification

Chopped paddy straw (1 cm pieces) having 35% cellulose, 22.2% hemicellulose and 3% moisture was used as substrate for saccharification was pretreated with steam. Erlenmeyer flasks, containing 5 g rice straw, were autoclaved at 15 psi pressure and 121°C temperature for 30 min followed by extraction with 0.1 N NaOH (in a 1:10 ratio of biomass sample to NaOH). It was followed by two washings with distilled water in the same ratio to bring down the pH and the biomass was

vacuum filtered. The washed biomass was then used as the substrate for saccharification.

Compositional analysis of pretreated rice straw

For compositional analysis the rice straw was dried to constant weight and moisture content was calculated. Hemicellulose content in the substrate was estimated by the TAPPI standard method [16] and the cellulose content of dried biomass samples was estimated by the procedure given by Updegraff [17].

Enzymatic hydrolysis of pretreated rice straw using standard cellulases supplemented with concentrated xylanase produced by *A. awamori* F18

Saccharification of steam-pretreated rice straw was carried out following the procedure given by Saritha et al. [11]. The substrate in wet form (1 g) with 85% moisture was taken in 50 ml plastic bottles to which different enzyme mixtures were added and volume was made up to 10 ml by adding 0.05 M citrate buffer (pH 4.8). The reaction mixture in bottles was incubated in a shaker water bath at 50°C and 0.5 ml aliquots were withdrawn periodically and reaction stopped by boiling the mixtures for 2 minutes. Total reducing sugars released during saccharification were estimated by the DNSA method and HPLC. All the treatments were run in triplicates. The different enzyme combinations used for saccharification of pretreated rice straw were:

- 0.5 ml Accelerase®1500 (Genencore)
- 0.1 ml Accelerase®1500 (Genencore)
- 1.0 ml concentrated xylanase
- 0.1 ml Accelerase®1500 (Genencore) + 1.0 ml concentrated xylanase
- 0.1 ml cellulase (Sigma)
- 0.1 ml cellulase (Sigma) + 1.0 ml concentrated xylanase
- 0.1 ml cellulase (Sigma) + 0.1 ml β glucosidase (Sigma)
- 0.1 ml cellulase (Sigma) + 0.1 ml β glucosidase (Sigma) + 1.0 ml concentrated xylanase

HPLC analysis of enzyme hydrolysate

The hydrolysates were analyzed for the presence of sugars using High Performance Liquid Chromatography (Waters 515 (Waters Corporation, Milford, MA, USA) equipped with a Waters 2414 Refractive Index Detector (RID)). The Aminex HPX-87H column was operated with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.5 ml min^{-1} and the oven temperature was kept at 40°C.

Results and Discussion

Xylanase production by hyperxylanolytic *A. awamori* F18 strain

Xylanolytic ability of *A. awamori* F18 strain was evident by its growth on Reese's mineral medium containing xylan as sole carbon source. It also produced hydrolysis zone on CMC agar indicating cellulase production. High levels of xylanase and cellulases were produced by *A. awamori* F18 under submerged culture with rice straw as substrate (Table 1). With 0.1% xylan, high xylanase (25.5 IU/ml), FPase and β-glucosidase activities were expressed after 5 days incubation and maximum CMCase was obtained after 7 days (Table 2). Most fungi are reported to produce a wide array of enzymes capable of depolymerizing plant cell wall polysaccharides, extracellularly [18]. Lignocellulosic substrates induce the production of cell wall degrading enzymes by bacteria and fungi under submerged and solid state conditions [19-21]. Most of the commercial cellulases are produced by the filamentous fungi - *Trichoderma reesei* or *Aspergillus niger* under submerged fermentation. As filamentous fungi have the tendency to grow adhered to surfaces and considering that submerged free floating fungal growth is not natural, favorable physiological aspects are associated with solid-state fermentation [22]. *A. awamori* F18 produced very high levels of xylanase on corncob substrate under solid state fermentation. Activities (IU g^{-1} of substrate) of different enzymes were 1432.09, 121.69, 19.06 and 86.80, for xylanase, CMCase, FPase and β-glucosidase, respectively (Table 3). Solid state fermentation technology has obvious advantages for production of commercial enzymes using fungi and lignocellulosic substrates as the cultivation conditions are suitable for growth of fungi which are able to grow at low water activities. Therefore, enzyme productivity in solid state fermentation is higher than that of submerged fermentation [23]. Many researchers have used agricultural and other wastes as substrates for production of cellulases and xylanases with this technology [20,24,25].

The enzyme activities were concentrated by precipitation with acetone and 11.5, 12.5 and 10.6 fold concentration of xylanase, CMCase and FPase, respectively, was achieved, with respective activity recoveries of 52.39%, 61.31% and 36.09% (Table 4). Simkhada et al. [26] produced crude xylanase enzyme in corn cob medium and obtained 4.7 fold purification and 23.6% activity recovery using a single step size exclusion chromatography.

Characterization of xylanase produced by *A. awamori* F18

To use different enzymes in combination, it is important that they have compatible activity parameters. Xylanase activity had an optimum temperature of 45°C and optimum pH of 6.0. Xylanases produced from *Aspergillus* strains were found to be active in a pH range of 5.0-7.0 with an optimum temperature from 40-70°C [27]. Most of the cellulases tried or recommended for bioconversion perform better at pH range 4-6 and at temperature ranging from 50 to 65°C; though at lower ends of the temperature range, the effective life time of enzyme is higher [22].

Xylanase	CMCase	FP activity	β-D-glucosidase
2.22 ± 0.109[x]	0.63 ± 0.04	0.09 ± 0.001	1.88 ± 0.10
(183.64)[I]	(46.96)	(6.91)	(140.98)

[x]Data represents the means ± SD, n = 3
[I]Values in brackets show the specific activity (IU/mg proteins)

Table 1: Enzyme activities (IU/ml) produced by *A. awamori* F18 on Reese's medium with paddy straw (1%) as sole carbon source under submerged fermentation for 7days.

Xylanase			CMCase			FP activity			β-D-glucosidase		
3 days	5 days	7 days	3 days	5 days	7 days	3 days	5 days	7 days	3 days	5 days	7 days
11.6 ± 4.28[x]	25.5 ±1.59	24.8 ± 2.10	0.3 ± 0.05	2.09 ± 0.2	2.37 ± 0.1	0.05 ± 0.001	0.07 ± 0.001	0.07 ± 0.001	0.14 ± 0.015	0.35 ± 0.04	0.35 ± 0.03

[x]Data represents the means ± SD, n = 3

Table 2: Enzyme activities (IU/ml) produced by *A. awamori* F18 on Reese's medium with xylan (0.1%) as sole carbon source under submerged fermentation.

Enzymatic saccharification

Enzymatic saccharification studies were carried out on steam-pretreated rice straw with different combinations of enzymes. Pretreated rice straw showed enriched cellulose (46%) and hemicellulose (27.5%) contents as compared to raw rice straw which had 35% and 22.2% cellulose and hemicelluloses, respectively on dry weight basis. It had moisture content of 85%. This enrichment in holocellulose is because of lignin removal during steam pretreatment. Different pretreatments result in enrichment of holocellulose by removing lignins/ hemicelluloses. Depending on severity of applied conditions, steam pretreatment typically results in significant depolymerisation of lignin component and extraction subsequently carried out after steam pretreatment will likely solubilize a significant fraction of this depolymerised lignin [28]. Saritha et al. [11] achieved enrichment of cellulose content resulting from delignification by biological pretreatment followed by alkali wash.

Activity (IU/g substrate)				
Substrate	Xylanase	CMCase	FP activity	β-D-glucosidase
5 g corn cob	1432.09 ± 17.17[x]	121.69 ± 3.23	19.06 ± 2.47	86.80 ± 11.52
	(456.19)[I]	(38.57)	(7.14)	(27.88)
100 g corn cob	856.03 ± 12.19	61.67 ± 4.01	8.12 ± 1.87	44.32 ± 3.05
	(524.43)	(43.95)	(8.64)	(29.81)

[x]Data represents the means ± SD, n = 3
[I]Values in brackets shows the specific activity of enzymes

Table 3: Enzyme activities produced by *A. awamori* F18 under solid state fermentation of corn cob for 7 days.

	Enzyme activity (IU/ml)			Proteins (mg/ml)	Specific activity (IU/mg proteins)		
	Xylanase	CMCase	FPase		Xylanase	CMCase	FPase
Crude extract	95.8	8.1	1.5	0.21	456.19	38.57	7.14
Extract after acetone precipitation	1110.9	101.38	15.9	0.42	2645	241.38	37.85
	% Recovery				Fold concentration		
	52.39	61.31	36.09		11.5	12.5	10.6

Table 4: Concentration of enzymes produced by *A. awamori* F18 under solid state fermentation on corn cob substrate.

Enzymatic saccharification of pretreated rice straw resulted in release of sugars in high amounts in all the treatments. Treatments in which concentrated *Aspergillus* xylanase was supplemented to standard cellulases resulted in significantly higher sugar yields as compared to respective controls. Accelerase®1500 (0.1 ml) supplemented with 1ml concentrated xylanase yielded 538.7 mg of sugars g^{-1} dry substrate (Figure 1a) which was 69.5% more than when Accelerase®1500 (0.1 ml) was used alone. However, the combination of concentrated xylanase, cellulase (Sigma) and β-glucosidase (Sigma) yielded the maximum sugars (629.7 mg g^{-1} dry substrate) after 72 hours (Figure 1b). Enzyme hydrolysate analyzed by HPLC showed significantly higher glucose release, and also release of xylose and arabinose in treatments receiving concentrated xylanase (Table 5). A 12.4% enhancement in the release of glucose and total sugars was evident from the results. Enhanced levels of total sugars as detected with DNSA method and also higher levels of different sugars as observed by HPLC showed that enhancement was there in degradation of cellulose and also hemicellulose was hydrolyzed as xylose was also present. This indicated that addition of xylanase enhanced total sugar recovery not only by degrading hemicelluloses but also facilitated enhanced hydrolysis of cellulose by cellulase enzymes. Saccharification of different pretreated agro residues by crude xylanase produced from *Aspergillus foetidus* MTCC4898 was attempted by Chapla et al. [29]. The maximum reducing sugars (mg g^{-1}) produced ranged from 151.6-193.86 and HPLC analysis of hydrolysates showed

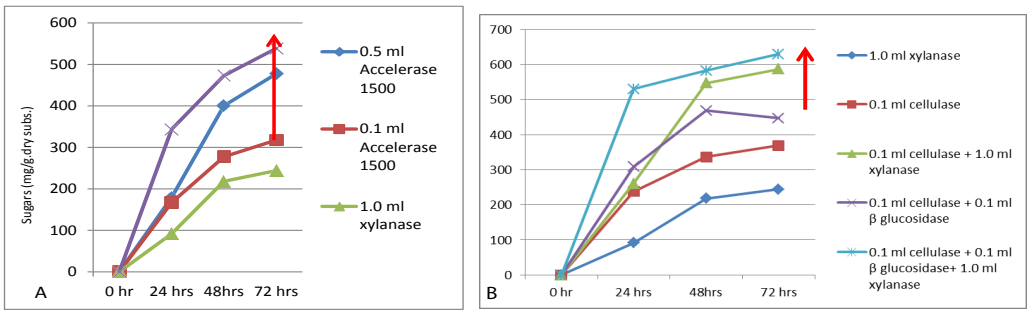

Figure 1: Enhancement of sugar yields by xylanase supplementation to (a) Accellerase®1500 (Genencore) (b) Cellulase (Sigma) and β-glucosidase (Sigma)

Treatment	Sugars(mg/g dry substrate)			Total sugars (mg/g dry substrate)
	Glucose	Xylose	Arabinose	
0.1 ml Accelerase®1500	105.33	14	0	119.33
0.1 ml cellulase (Sigma) + 0.1 ml β-glucosidase (Sigma)	256.66	58	178	492.66
0.1 ml cellulase (Sigma) + 0.1 ml β-glucosidase (Sigma) + 1.0 ml conc. Xylanase	288.66	68	196.66	553.33
SE(m) ±				1.702
CD@5%				9.45

Table 5: Different sugars present in hydrolysates detected by HPLC.

xylose to be the major end product with only traces of glucose. Hu et al. [30] also reported an enhancement in enzyme hydrolysis of steam pretreated corn stover when xylanase were added to minimum amount of cellulase used for hydrolysis. The co-hydrolysis of xylan increased accessibility of cellulose to cellulases. Apparently supplementing the cellulases with xylanases increased both the cellulose and hemicelluloses hydrolysis. This effect holds true for all pretreatments with the benefit of xylanase supplementation depending on type of pretreatment with total sugar yield boost between 40-100% [8].

Hemicelluloses may add to the recalcitrance of cellulose and enzymes such as xylanase are common in industrial enzyme cocktails for lignocellulose processing [31]. Rice straw pretreated with a GH11 endoxylanase from *Chaetomium globosum* showed a decrease in hemicellulose content from 25.8 to 11.4% and an increased saccharification rate [9]. This was due to the hydrolysis of hemicelluloses, thereby exposing a more accessible surface area of cellulose to cellulase. While role and synergistic actions of classical endoglucanases and cellobiohydrolases in degradation of cellulose are well understood, conversion yields with these are well below 100% [32]. Presence of other enzymes in the cocktails and their role in increasing the efficiency of cellulases by increasing accessibility of cellulose is important. One of the main beneficial effects of cellulase supplementation with xylanase during biomass saccharification is thought to be the result of improved cellulose accessibility as a result of xylan solubilization [8]. To use lignocellulosic biomass industrially, it is important that cellulase and hemicellulase work in concert. The hemicellulose-degrading enzyme activities in most of the commercial cellulase preparations are low to achieve significant conversion of the residual hemicellulose [33]. Therefore, supplementation of cellulase with the so-called accessory enzymes, including xylanases, has been the most common approach to increase the overall fermentable sugar yields [30]. There are definite reports that xylanase supplementation in combination with pretreatment or cellulases during saccharification enhance the yields of sugars [8,34]. An analysis of the saccharification ability of high-functional cellulase for various pretreated biomass by Kawai et al. [10] showed that commercial enzymes with enhanced cellobiase activity gave low glucose yields as the remaining xylan inhibited the degradation of cellulose. Therefore, they found that addition of a hemicellulase to Accelerase®1500 increases not only xylose yield but also glucose yield. Benefits of xylanase addition during pretreatment of wheat straw and rice straw have resulted in enhanced sugar yields [8,9]. In the present study, xylanase was supplemented to commercial biomass hydrolyzing cellulases during saccharification step and there was 12.4% enhancement in total sugar yields. The results of the study, therefore, confirmed that accessory xylanase improved saccharification of pretreated rice straw by cellulases and enhanced sugar yields. Accessory enzymes that facilitate more complete utilization of plant biomass could be used to develop less energetically and chemically intensive processing and allow for greater fermentable sugar recovery. Thus, enzyme mixtures of the future comprising accessory enzymes need to be tailored for essential lignocellulolytic activities to achieve improved overall performance yields.

Conclusions

In coming years bio refineries will form key components of sustainable bio based economy and conversion of polysaccharides present in biomass with enzymes will be an important technology. The present study showed that to realize higher sugar yields and conversion of both the constituent polysaccharides of lignocellulosic biomass, a mixture of enzymes is required and is more efficient. Results of this study demonstrate the role of accessory enzyme xylanase in enhancing saccharification efficiency of lignocellulosic biomass.

Authors' contributions

Jairam Choudhary conducted the experiments. Jairam Choudhary and Saritha M wrote the manuscript. Anju Arora and Lata Nain conceptualized and critically reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgement

Authors are thankful to Dr. S. Singh for HPLC analysis of hydrolysates. Jairam Choudhary and Saritha M are thankful to the Post Graduate School and Director, Indian Agricultural Research Institute, New Delhi, India and the Department of Science and Technology, GOI for providing for fellowship. We are also grateful to the Division of Microbiology, IARI, New Delhi for providing necessary facilities for undertaking this study.

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