CIBTech Journal of Microbiology ISSN: 2319-3867 (Online)
An Online International Journal Available at http://www.cibtech.org/cjm.htm
2015 Vol. 4 (3) July-September, pp.7-13/Shankarappa et al.
Research Article

ENZYMATIC CONVERSION OF ALKALI PRETREATED AGRORESIDUES TO FERMENTABLE SUGARS BY COMMERCIAL CELLULASE

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ABSTRACT
The use of commercial cellulase enzyme with 15 U per g concentration along with β-glucosidase and Xylanase enzymes at 10 and 5 U per g, respectively on alkali pre-treated substrates (Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover) at 5% substrate concentration was found to be optimum for higher saccharification. This combination produced significantly the highest reducing sugars release and per cent saccharification respectively in sugarcane bagasse (655.32 mg g^{-1} and 93.17%), sugarcane tops (615.23 mg g^{-1} and 90.33%), sugarcane trash (576.28 mg g^{-1} and 84.61%), corn husk (555.30 mg g^{-1} and 80.61%) and in corn stover (550.37 mg g^{-1} and 80.80%) in 12 h incubation period. The use of 20 U per g commercial cellulase enzyme concentration along with β-glucosidase (10 IU g^{-1}) and Xylanase (5 U g^{-1}) or extension of incubation period up to 24 h stood on par with 15 U per g commercial cellulase enzyme concentration along with β-glucosidase (10 IU g^{-1}) and Xylanase (5 U g^{-1}) and 12 h incubation in releasing the reducing sugars.

Keywords: *Agroresidues, Alkali Pretreatment, Commercial Enzyme, Enzyme Concentration, Saccharification*

INTRODUCTION
Several raw materials are being used for the bioethanol production *viz*. easily fermentable sugary feed stocks (molasses), starchy feed stocks (grains) and feed stocks containing complex sugars in the form of cellulose and hemicellulose. Sugar materials, starchy materials are in the human food chain and are thus expensive. Therefore, the next alternative is cellulosic materials. Several feed stocks have been studied for their potentiality to yield fermentable sugars to produce ethanol by various researchers *viz*. rice straw (Karimi *et al*., 2006), Bagasse (Singhania *et al*., 2006), Cotton stalks, (Kerem *et al*., 1992), Wheat straw (Zayed and Meyer, 1996), Alfalfa fibre (Sreenath *et al*., 2001), Sugar cane leaves (Harikrishna *et al*., 2001), sun flower hulls (Sharma *et al*., 2004) and Corn stover (Teymouri *et al*., 2005).
The abundantly available lignocelluloses require pre-treatment for obtaining fermentable sugars and conversion of the same to ethanol. There are numerous pre-treatment methods or combinations of pre-treatment methods available. The most common one is the chemical pre-treatment that uses alkali, NaOH (Shankarappa and Geeta, 2013), to make the biomass more digestible by the enzymes (Schell *et al.,* 2003; Mosier *et al.,* 2005), so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields.
The hydrolysis of cellulose and hemicelluloses polysaccharides in to their respective monomers is called as saccharification, it involves cellulolytic microorganisms or their enzymes namely, cellulase, hemicellulase and xylanases. Three major types of enzymatic activities are found in cellulase system: (i) endoglucanases (EC 3.2.1.4), (ii) exoglucanases (EC 3.2.1.74) and cellobiohydrolases (EC 3.2.1.91) and (iii) β -glucosidases (EC 3.2.1.21). Endoglucanases cut, at random, at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends (Bisaria and Ghose, 1981). Exoglucanases act on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose by

peeling cellulose chains from the microcrystalline structure. The β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose.

Therefore, in this study, the abundantly available agroresidues which are outside the human food *viz.* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were pretreated with alkali for delignification followed by enzymatic saccharification using commercial cellulase enzymes to recover fermentable sugars to be subjected for alcohol fermentation.

MATERIALS AND METHODS

Five substrates *viz.*, sugarcane bagasse (procured from Malaprabha sahakari sugar factory, M. K. Hubli, Belgaum, Karnataka, sugarcane trash (Co-8014) and sugarcane tops (Co-8014) from the fields of Mr. Basavaraj, Yettinagudda, Dharwad, Karnataka and corn stover (Arjun) and corn husk (Arjun) from Main Agricultural Research Station, University of Agricltural Sciences, Dharwad, Karnataka were brought to the laboratory, chopped into small pieces, dried at 60^0C in a hot air oven for 12 h and powdered by dry milling (Willey mill) to obtain particle size of 0.5 mm (Karimi *et al.*, 2006).

The substrates *viz*. Sugarcane bagasse, Sugarcane tops, Sugarcane trash, Corn husk and Corn stover of particle size 0.50 mm were pretreated with alkali NaOH @ 3.0% for 8 hours under ambient condition and autoclaved at 121^0C, 15 lbs pressure for 1h. The quantity of alkali used was approximately 50 ml 10 g^{-1} dry substrate taken in 250 ml Erlenmeyer flasks, which was sufficient enough to moisten entire substrate except in case of bagasse where additional 10 ml was used (Shankarappa and Geeta, 2013). After the alkali and heat pre-treatment, substrates were washed with tap water followed by distilled water to remove the alkali content (until the pH was close to 7.0). Otherwise, the pH of the substrates was neutralized with acetic acid. The residue obtained after the treatment was dried in a hot air oven at 60^0C to constant weight. The delignified agroresidues had cellulose content of 0.633 g g^{-1} in sugarcane bagasse, 0.613 g g^{-1} in sugarcane tops, 0.613 g g^{-1} in sugarcane trash, 0.620 g g^{-1} in corn husk, and 0.613 g g^{-1} in corn stover (Shankarappa and Geeta, 2013).

The pre-treated substrates *viz*., sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were hydrolysed by using commercial cellulase and β–glucosidase and xylanase enzymes. Oven dried, alkali and temperature pre-treated samples (5.0 g) were suspended separately in 250 ml Erlenmeyer flask containing varied quantities of 0.05 M Citrate buffer, pH 4.8 (autoclaved and added with 10, 15 and 20 FPU g^{-1} cellulase enzyme (MAPS India, Ahmadabad) separately. The β–glucosidase (SRL chemicals) enzyme at 10 IU g^{-1} and xylanase enzyme at 5 U g^{-1} (M/s. Sigma Industry – Courtesy, Godavari Sugar Mills, Sameerwadi) were also supplemented commonly making the ratio at 10:10:5, 15:10:5 and 20:10:5 for cellulase, β-glucosidase and xylanase, respectively to enhance the rate of cellulose hydrolysis and to overcome the inhibition of glucose formation by cellobiose (Sattler *et al.*, 1989; Sun and Cheng, 2005). The final volume of buffer and enzyme mixture was 100 ml solution. The cellulose enzyme of M/s. MAPS India, Ahmedabad had cellulase activity of 166 FPU ml^{-1}, the β–glucosidase of M/s. SRL Chemicals had β–glucosidase activity of 19.6 U mg^{-1} and the xylanase of M/s. Sigma Chemicals had xylanase activity of 170 U g^{-1}. The suspension was incubated for varying intervals of time 0 to 24h at 50^0 C at 150 rpm. Then the reaction of hydrolysis was ceased to proceed by holding flasks in boiling water for 10 min to denature the enzyme. Solution of 4 ml was withdrawn, centrifuged at 10000 rpm for 10 min and the supernatant was used for the estimation of reducing sugars and per cent hydrolysis (Kaar and Holtzapple, 1998). The amount of reducing sugars was estimated by Di-Nitro Salicylic Acid (DNSA) method (Miller, 1959). The per cent saccharification was calculated by the formula given below.

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars (mg g}^{-1}) \times 0.9 \times 100}{\text{Initial cellulose (mg g}^{-1})}$$

RESULTS AND DISCUSSION

The alkali pretreated agroresidues, 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 121^0C (1 h) had cellulose content of 0.633 g g^{-1} in sugarcane bagasse, 0.613 g g^{-1} in sugarcane tops, 0.613

g g^{-1} in sugarcane trash, 0.620 g g^{-1} in corn husk, and 0.613 g g^{-1} in corn stover. These delignified substrates were used for saccharification studies using different cellulolytic fungi (Shankarappa and Geeta, 2013).

Table 1: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane bagasse

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	184.38 (26.21)	305.29 (43.41)	396.35 (56.35)	8.29 (1.18)	**223.58**
4	296.23 (42.12)	420.44 (59.78)	509.00 (72.37)	8.32 (1.18)	**308.50**
8	346.48 (49.26)	541.59 (77.00)	610.25 (86.77)	8.36 (1.19)	**376.67**
12	361.51 (51.39)	655.32 (93.17)	654.24 (93.02)	8.38 (1.19)	**419.86**
24	364.33 (51.80)	658.41 (93.61)	658.05 (93.56)	8.39 (1.19)	**422.30**
Mean	**310.59**	**516.21**	**565.58**	**8.35**	
	SE±		**CD (1%)**		
Incubation time (A)	1.271		4.863		
Concentrations of enzyme (B)	1.137		4.350		
Interaction (A x B)	2.543		9.726		

Figures in parentheses indicate per cent saccharification
Note: Initial cellulose content 633 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 2: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane tops

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	178.48 (26.20)	301.17 (44.22)	371.38 (54.52)	8.26 (1.21)	**214.83**
4	284.52 (41.77)	388.49 (57.04)	480.64 (70.56)	8.40 (1.24)	**290.51**
8	321.68 (47.23)	520.53 (76.43)	579.41 (85.10)	8.40 (1.23)	**357.51**
12	348.54 (51.17)	615.23 (90.33)	615.89 (90.42)	8.41 (1.24)	**397.02**
24	350.44 (51.45)	616.37 (90.49)	618.35 (90.79)	8.45 (1.24)	**398.40**
Mean	**296.73**	**488.36**	**533.14**	**8.39**	
	SE±		**CD (1%)**		
Incubation time (A)	1.474		5.636		
Concentrations of enzyme (B)	1.318		5.041		
Interaction (A x B)	2.947		11.272		

Figures in parentheses indicate per cent saccharification
Note: Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 3: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane trash

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	167.45 (24.59)	286.56 (42.07)	350.90 (51.52)	8.24 (1.21)	**203.29**
4	258.38 (37.94)	380.58 (55.87)	472.43 (69.36)	8.26 (1.21)	**279.91**
8	302.51 (44.41)	500.49 (73.48)	543.54 (79.80)	8.35 (1.23)	**338.72**
12	327.50 (48.08)	576.28 (84.61)	574.34 (84.32)	6.40 (0.94)	**371.13**
24	330.44 (48.51)	578.34 (84.91)	576.34 (84.62)	8.42 (1.24)	**373.38**
Mean	**277.26**	**464.45**	**503.51**	**7.94**	
	SE±		**CD (1%)**		
Incubation time (A)	1.316		5.034		
Concentrations of enzyme (B)	1.177		4.503		
Interaction (A x B)	2.633		10.069		

Figures in parentheses indicate per cent saccharification
Note: Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 4: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from corn husk

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	163.33 (23.71)	273.49 (39.70)	348.04 (50.52)	8.25 (1.20)	**198.28**
4	253.51 (36.80)	365.43 (53.05)	471.37 (68.42)	8.27 (1.20)	**274.65**
8	300.37 (43.60)	480.47 (69.75)	544.45 (79.03)	8.33 (1.21)	**333.41**
12	324.47 (47.09)	555.30 (80.61)	556.55 (80.79)	8.36 (1.21)	**361.17**
24	329.43 (47.82)	558.38 (81.10)	560.25 (81.33)	8.43 (1.22)	**364.12**
Mean	**274.22**	**446.61**	**496.13**	**8.33**	
	SE±		**CD (1%)**		
Incubation time (A)	1.074		4.108		
Concentrations of enzyme (B)	0.961		3.674		
Interaction (A x B)	2.148		8.215		

Figures in parentheses indicate per cent saccharification
Note: Initial cellulose content 620 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 5: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from corn stover

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	143.56 (21.07)	273.49 (40.15)	346.56 (50.88)	8.24 (1.21)	**192.96**
4	235.20 (34.53)	365.47 (53.66)	452.36 (66.41)	8.29 (1.23)	**265.33**
8	326.40 (47.92)	482.44 (70.83)	502.52 (73.78)	8.33 (1.22)	**329.93**
12	348.32 (51.14)	550.37 (80.80)	550.24 (80.79)	8.36 (1.23)	**364.32**
24	350.63 (51.48)	553.41 (81.25)	554.23 (81.37)	8.38 (1.23)	**366.66**
Mean	**280.82**	**445.04**	**481.18**	**8.32**	
	SE±		**CD (1%)**		
Incubation time (A)	1.310		5.010		
Concentrations of enzyme (B)	1.172		4.481		
Interaction (A x B)	2.620		10.020		

Figures in parentheses indicate per cent saccharification
Note: Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

The alkali pretreated substrates *viz*. Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover studied for saccharification at 5% substrate concentration with different concentrations of cellulase enzyme *viz*. 10, 15 and 20 FPU g-$^{-1}$ cellulase enzyme along with β–glucosidase at 10 IU g^{-1} and xylanase at 5 U g^{-1} in the ratio at 10:10:5, 15:10:5 and 20:10:5 for cellulase, β-glucosidase and xylanase indicated variations in the amount of release of reducing sugars.

The different concentrations of commercial cellulase enzymes indicated marked variations with regard to release of reducing sugars. Significantly the mean maximum reducing sugar release, 565.58, 633.14, 503.71, 496.13 and 481.18 mg per g was recorded for 20 U of enzyme concentration, which was significantly superior over 15 U and over 10 U enzyme concentrations.

The release of reducing sugars over different periods of incubation varied significantly. The mean higher release of reducing sugars, 422.30, 398.40, 373.38, 364.12 and 366.66 mg per g was recorded at the end of 24 h incubation period and was found to be on par with 12 h incubation period (419.87, 397.02, 371.13, 361.17 and 364.32 mg g^{-1}) respectively in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover (Tables 1, 2, 3, 4 and 5). 24 h and 12 h incubations were significantly superior over 8 h, 4 h and over 2 h incubation periods with respect to release of reducing sugars.

The combined effect of different concentrations and incubation period on release of reducing sugars differed significantly in all the pre-treated substrates. The commercial cellulase when used at 15 U per g in 12 h reaction time yielded significantly superior amounts of reducing sugars and per cent saccharification respectively in sugarcane bagasse (655.32 mg g^{-1} and 93.17%), sugarcane tops (615.23 mg g^{-1} and 90.33%), sugarcane trash (576.28 mg g^{-1} and 84.61%), corn husk (555.30 mg g^{-1} and 80.61%) and in corn stover (550.37 mg g^{-1} and 80.80%). Kumar *et al.*, (2010) reported similar kind of observations with steam pre-treated substrates where use of 20 FPU per g cellulase resulted in almost 90 per cent saccharification within 12 h of incubation. The sugar release and percent saccharification although found fractionally more when incubated up to 24 h either with 15 FPU g-$^{-1}$ cellulase or with 20 FPU g-$^{-1}$ cellulase, it was not found to be superior to 12 h incubation with 15 FPU g-$^{-1}$ cellulase (Tables 1, 2, 3, 4 and 5). Sugarcane bagasse and sugarcane tops are found to be superior substrates for sugar recovery over other tested substrates.

The release of reducing sugars as influenced by various concentrations of commercial cellulase enzymes showed that the inoculation of 15 U per g was optimum for higher saccharification of the pre-treated substrates. The data outlined in Table 2, 3, 4, 5 and 6 reveal that the use of 15 U per g enzyme concentrations had resulted in higher amounts of reducing sugar release at 12 h incubation period in all the pre-treated substrates. The extension of incubation period up to 24 h with 15 U per g enzyme concentration did not increase the release of reducing sugars in significant amounts since the adsorption site, pore size and accessibility of the substrate for enzyme hydrolysis would have decreased after 12h reaction period and also the released sugars would have inhibited the enzyme for further hydrolysis (Sattler *et al.*, 1989).

It was also observed that the release of reducing sugars were significantly highest with higher concentrations of enzyme (20 U) in the initial stages but the saccharification had reached saturation point at 12 h incubation in release of reducing sugars and found on par with 15 U enzyme concentration at 12 h incubation. These results suggest that the optimum commercial cellulase enzyme concentration required for saccharification of different pre-treated substrates was 15 U per g substrate.

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