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Saccharification of alkali pretreated agroresidues to fermentable sugars by crude enzymes of cellulolytic fungi

Thyavanahally Hanumaiah Shankarappa[1]*, Gurunath Shirnalli Geeta[2], Mudigere Javaraiah Manju[1], Chandragouda Rudragouda Patil[2], Hirematha Vamadevaiah[2], Kavi Shivalingappa Jagadeesh[2]

1 Department of Agricultural Microbiology, College of Horticulture, Tamaka, Kolar, 563103, India.
2 Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad- 580 005, India

E-mail : shankarappath@gmail.com Contact No. : 632-8372071 loc. 2216

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Abstract

Five agro-residues *viz*. sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were alkali pretreated using 3.0% NaOH for 8 hours under ambient condition and then autoclaved at 121^{0}C, 15 lbs pressure for 1h. The pretreated residues were subjected for saccharification using crude enzymes of four proven cellulolytic fungi at 10, 20 and 30 FPU per gram substrate. The release of reducing sugars as influenced by various concentrations of crude cellulase enzymes indicated that the treatment of 20U g^{-1} was optimum for all the crude enzyme extracts of different fungi studied. The crude enzyme extract of *Trichoderma reesei* when used at 20 U g^{-1} showed the significantly superior amounts of release of reducing sugars and per cent saccharification respectively in sugarcane bagasse (477.45 mg g^{-1} and 67.88%), sugarcane tops (428.68 mg g^{-1} and 62.94%), sugarcane trash (437.45 mg g^{-1} and 64.22%), corn husk (411.26 mg g^{-1} and 59.70%) and in corn stover (421.83 mg g^{-1} and 61.32%).

Key words : Agroresidues, Crude enzyme, Cellulolytic fungi, saccharification

INTRODUCTION

The most common renewable fuel produced today is ethanol derived from corn grain (starch) and sugar cane (sucrose). It is expected that there will be limits to the supply of these raw materials in the near future; therefore, lignocellulosic biomass is seen as an attractive feed stock for future supplies of ethanol [1]. The abundantly available lignocelluloses require pre-treatment for obtaining fermentable sugars and conversion of the same to ethanol. Several pretreatment methods have been used for effective delignification and recovery of cellulose and hemicellulose sugars from various substrates that includes physical, chemical [2], combination of physical and chemical [3] and biological pretreatments [4].

The hydrolysis of cellulose and hemicelluloses polysaccharides in to their respective monomers called as saccharification involves cellulolytic microorganisms or their enzymes namely, cellulase, hemicellulase and xylanases. Several fungi are known to produce extracellular enzymes and bring about saccarification namely, *Trichoderma reesei* [5], *T. Viridae* [6], *Aspergillus awamorii* [5], *Phanerochaete chrysosporium* [7] etc. The commercial enzymes required to be used for saccharification is expensive and the process is not viable, in this regard the use of inexpensive crude enzymes of certain fungi is a very good prospect.

Several feed stocks have been studied for their potentiality to yield fermentable sugars to produce ethanol by various researchers *viz*. rice straw [8], Bagasse [9], Cotton stalks, [10], Wheat straw [6], Alfalfa fibre [11], Sugar cane leaves [12], sun flower hulls [13] and Corn stover [14]. 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 crude enzymes of proven, efficient cellulolytic fungi 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^{0}C in a hot air oven for 12 h and powdered by dry milling (Willey mill) to obtain particle size of 0.5 mm [8]. Crude enzymes extracted from five, proven cellulolytic fungal cultures *viz. Trichoderma reesei, T. viridae, Aspergillus awamorii, A. sydowii* and *Phanerochaete chrysosporium* obtained from Department of Agricultural Microbiology, University of Agricltural Sciences, Dharwad, Karnataka were used for saccharification studies.

The five different fungal cultures were inoculated separately in to mandal's basal salt solution [15] for cellulase enzyme production. The individual cultures were inoculated to the 100 ml of autoclaved basal salt solution @ 5%, taken in 250 ml Erlenmeyer flask incubated for eight days on rotary shaker at 150 rpm at 30^0 C at 6.0 pH on cellulose as carbon source, since these conditions produced highest cellulase activity for all the cellulolytic cultures. After eight days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant was collected and used as the source of enzyme. The culture filtrates obtained from eight days grown fungi at 30^{0}C and pH 6.0 had different enzyme activities as shown in Table 1 [16].

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^{0}C, 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 [2]. 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^{0}C 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 [2].

The pre-treated substrates (Sugarcane bagasse, Sugarcane tops, Sugarcane trash, Corn husk and Corn stover) were subjected for crude enzyme hydrolysis in 250 ml Erlenmeyer flask. Oven dried, alkali and temperature pre-treated samples (5.0 g) were suspended in varying quantities of 0.05 M Citrate buffer of pH 4.8 such that after the addition of enzyme extract the final volume of solution was 100 ml. [17, 7]. The substrate and buffer solution was autoclaved at 121^0 C for 15 min and added with crude enzyme extract of different cellulolytic fungi. The final volume of buffer and enzyme extract was 100 ml solution. The suspension was incubated for varying intervals of time 0 to 48 h 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 10,000 rpm for 10 min and the supernatant was used for the estimation of reducing sugars and per cent hydrolysis.

The crude enzymes extracted from different fungi had varying degrees of activities for FPU activity, CMCase activity, β-glucosidase activity, xylanase activity and protein content in the extract as indicated in Table 1. The crude enzyme extracts *viz*. *Trichoderma reesei, T. viridae, Aspergillus awamorii, A. sydowii* and *Phanerochaete chrysosporium* were used separately @ 10, 20 and 30 FPU g-^{1} with 5% substrate concentration [16].

The amount of reducing sugars was estimated by DNSA method as described by Miller [18]. The per cent saccharification [19] was calculated by the formula given below.

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

RESULTS

The alkali pretreated agroresidues, 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 121^{0}C (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 [2].

The alkali pretreated substrates *viz.* Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were subjected for saccharification at 5% substrate concentration using different concentrations of crude cellulase enzymes (10, 20 and 30 U) extracted from 4 different fungi (*T. viridae*, *T. reesei*, *A. sydowii* and *A. awamorii*). The use of different concentrations of crude enzymes expressed their activity differently with regard to release of reducing sugars.

The crude enzyme extract of fungi *Trichoderma reesei* produced the mean maximum release of reducing sugars of 364.84, 328.81, 340.40, 329.73 and 325.43 mg g^{-1} respectively in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and in corn stover which was significantly superior over other fungi. The lowest release of reducing sugars was observed in the control treatment which received no fungi inoculation (Tables 2, 3, 4, 5 and 6).

The release of reducing sugars as influenced by enzyme concentration indicated the mean maximum release of reducing sugars with 30 U recorded 307.90, 291.15, 297.61, 285.08 and 283.86 mg g^{-1} respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and for corn stover. It was significantly superior over 20 U and 10 U of FPU g^{-1} of substrate to release reducing sugars respectively in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and in corn stover.

As regards average incubation time, incubation up to 48 h resulted in highest release of reducing sugars respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and for corn stover (323.04, 298.59, 308.27, 298.26 and 294.63 mg g^{-1}) however, it was found to be on par with 24 h incubation time (322.17, 300.64, 308.09, 296.33 and 295.14 mg g^{-1} respectively in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and in corn stover). Incubation up to 24 h was found to be optimum to release maximum reducing sugars.

DISCUSSION

The release of reducing sugars as influenced by various concentrations of crude cellulose enzymes indicated that the inoculation of 20U g^{-1} was optimum for all the crude enzyme

Table 1: Enzyme activity of crude extract obtained from different cellulolytic fungi.

Cellulolytic fungi	FPU activity (U ml^{-1} min^{-1})	CMCase activity (U ml^{-1} min^{-1})	β-glucosidase activity (IU ml^{-1} min^{-1})	Xylanase activity (U ml^{-1} min^{-1})	Protein content (mg^{-1} ml^{-1})
Trichoderma viridae	2.21	2.79	1.56	2.75	**0.475**
Trichoderma reesei	2.33	3.27	1.82	3.42	**0.492**
Aspergillus sydowii	1.65	2.55	0.55	0.00	**0.511**
Aspergillus awamorii	1.52	2.41	0.45	0.00	**0.565**
Phanerochaete chrysosporium	1.33	2.38	0.34	1.03	**0.399**

Table 2: Effect of different concentrations of crude enzymes on release of reducing sugars in sugarcane bagasse (mg/g)

Treatments (A)	Enzyme conc. (C)	Fungal crude enzymes (B)					Means for treatments (A)
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowii*	*Aspergillus awamorii*	Control	
6 hours	10 U	149.83 (21.30)	183.35 (26.07)	157.83 (22.44)	146.02 (20.76)	8.29 (1.15)	178.26
	20 U	243.54 (34.63)	266.40 (37.88)	204.49 (29.08)	231.73 (32.95)	8.33 (1.18)	
	30 U	260.30 (37.01)	293.06 (41.67)	267.92 (38.09)	243.93 (34.68)	8.81 (1.25)	
12 hours	10 U	220.31 (31.32)	270.59 (38.47)	224.12 (31.87)	211.93 (30.13)	8.28 (1.15)	258.45
	20 U	347.16 (49.36)	357.45 (50.82)	344.11 (48.92)	352.49 (50.12)	8.71 (1.24)	
	30 U	370.40 (52.66)	403.54 (57.38)	368.88 (52.45)	379.92 (54.02)	8.85 (1.26)	
24 hours	10 U	312.49 (44.43)	341.83 (48.60)	318.21 (45.24)	303.73 (43.19)	8.66 (1.23)	322.17
	20 U	445.16 (63.29)	477.45 (67.88)	438.59 (62.36)	394.40 (56.07)	8.90 (1.26)	
	30 U	428.60 (60.94)	477.07 (67.83)	427.16 (60.73)	441.30 (62.75)	8.93 (1.27)	
48 hours	10 U	315.54 (44.86)	344.88 (49.03)	323.16 (45.95)	304.50 (43.29)	9.04 (1.29)	323.04
	20 U	451.93 (64.25)	480.85 (68.37)	440.87 (62.68)	396.30 (56.35)	9.09 (1.29)	
	30 U	432.46 (61.49)	481.64 (68.48)	442.78 (62.96)	403.54 (57.38)	8.98 (1.28)	
Means for Fungal crude enzymes (B)		**331.48**	**364.84**	**329.84**	**317.48**	**8.74**	

Means enzyme conc. (C)	10 U	208.13
	20 U	295.40
	30 U	307.90
	SEm ±	**CD 1%**
Treatments (A)	1.399	5.177
Crude enzymes (B)	1.564	5.788
Enzyme concentration (C)	1.211	4.483
A x B	3.127	11.576
A x C	2.422	8.966
B x C	2.708	10.025
A x B x C	5.417	20.050

Figures in parentheses indicate per cent saccarification
Note : Initial cellulose content 633 mg g^{-1}

extracts of different fungi studied. As per the data presented in Table 2, 3, 4, 5 and 6 the use of 20 U g-1 enzyme concentrations had resulted in maximum release of reducing sugar in 24 h incubation period. The extension of incubation period up to 48 h with 20 U g^{-1} enzyme concentration did not release significant amounts of reducing sugars.

The crude enzyme extract of *T. reesei* when used at 20 U g^{-1} showed the significantly superior amounts of release of reducing sugars and per cent saccharification respectively in sugarcane bagasse (477.45 mg g^{-1} and 67.88%), sugarcane tops (428.68 mg g^{-1} and 62.94%), sugarcane trash (437.45 mg g^{-1} and 64.22%), corn husk (411.26 mg g^{-1} and 59.70%) and in corn stover (421.83 mg g^{-1} and 61.32%). The present results are supported by the observations of Hatakka [20], who reported 41% reducing sugars yield after enzymatic saccharification with *Trichoderma reesei* cellulase from alkali pre-treated wheat straw (2%, NaOH w/v) at 115^0 C for 10 min. Further, the inoculation of substrates with *T. reesei* fungi yielded the highest reducing sugar release as

Table 3: Effect of different concentrations of crude enzymes on release of reducing sugars in sugarcane tops (mg/g)

Treatments (A)	Enzyme conc. (C)	Fungal crude enzymes (B)					Means for treatments (A)
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowii*	*Aspergillus awamorii*	Control	
6 hours	10 U	144.88 (21.27)	162.40 (23.85)	211.54 (31.06)	134.21 (19.70)	8.29 (1.22)	174.19
	20 U	229.45 (33.69)	240.87 (35.36)	227.92 (33.46)	219.55 (32.23)	8.52 (1.25)	
	30 U	247.35 (36.31)	266.92 (39.19)	238.69 (35.04)	263.35 (38.66)	8.83 (1.30)	
12 hours	10 U	245.83 (36.09)	211.54 (31.06)	211.54 (31.06)	203.16 (29.83)	8.28 (1.22)	242.48
	20 U	317.98 (46.69)	330.50 (48.52)	323.16 (47.45)	307.16 (45.10)	8.69 (1.28)	
	30 U	384.21 (56.41)	362.07 (53.16)	362.07 (53.16)	352.11 (51.70)	8.86 (1.30)	
24 hours	10 U	302.97 (44.48)	319.35 (46.89)	293.27 (43.06)	290.74 (42.69)	8.28 (1.22)	300.64
	20 U	396.30 (58.18)	428.68 (62.94)	380.68 (55.89)	385.64 (56.62)	8.90 (1.31)	
	30 U	460.69 (67.64)	434.78 (63.83)	403.16 (59.19)	387.16 (56.84)	8.91 (1.31)	
48 hours	10 U	304.49 (44.70)	321.64 (47.22)	296.96 (43.60)	295.35 (43.36)	9.04 (1.33)	298.59
	20 U	405.07 (59.47)	431.35 (63.33)	387.54 (56.90)	384.49 (56.45)	9.09 (1.33)	
	30 U	406.97 (59.75)	435.54 (63.94)	394.40 (57.91)	387.92 (56.95)	8.96 (1.32)	
Means for Fungal crude enzymes (B)		**320.52**	**328.81**	**310.91**	**300.91**	**8.72**	
Means enzyme conc. (C)		10 U			199.19		
		20 U			271.58		
		30 U			291.15		
		SEm ±			**CD 1%**		
Treatments (A)		0.667			2.468		
Crude enzymes (B)		0.745			2.759		
Enzyme concentration (C)		0.577			2.137		
A x B		1.491			5.519		
A x C		1.155			4.275		
B x C		1.291			4.779		
A x B x C		2.582			9.559		

Figures in parentheses indicate per cent saccarification
Note : Initial cellulose content 613 mg g^{-1}

compared to other cellulolytic fungal inoculation in respective pretreated substrates, suggesting that *T. reesei* is very efficient over other fungi in terms of sugar conversion due to production of various cellulolytic enzymes [5,7].

The inoculation of 20 U per gram, *T. reesei* crude enzyme after 24 h incubation did not increase the reducing sugars significantly when the incubation period was extended up to 48 h. Further, the use of 30 U per gram enzyme concentration for 24 h or 48 h incubation also failed to increase the reducing sugars beyond that produced by 20 U g^{-1} enzyme concentrations. The 20 U and 30 U enzyme concentrations although are different they produced on par saccharification and they attained saturation levels almost equal for a given pretreated substrate, 68 per cent in sugarcane bagasse, 63 per cent in sugarcane tops, 64 per cent in sugarcane trash, 61 per cent in corn husk and 61 per cent in corn stover. It could be due to inhibition of cellulase activity by the reducing sugars produced and other derivatives released by the crude enzyme extract. Holtzaple *et al*. [21] had reported the inhibition of

Table 4: Effect of different concentrations of crude enzymes on release of reducing sugars in sugarcane trash (mg/g)

Treatments (A)	Enzyme conc. (C)	Fungal crude enzymes (B)					Means for treatments (A)
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowii*	*Aspergillus awamorii*	Control	
6 hours	10 U	150.21 (22.05)	172.69 (25.35)	161.25 (23.68)	138.40 (20.32)	8.28 (1.22)	177.04
	20 U	226.02 (33.18)	243.16 (35.70)	243.16 (35.70)	231.35 (33.97)	8.52 (1.25)	
	30 U	261.07 (38.33)	280.12 (41.13)	252.30 (37.04)	270.21 (39.67)	8.82 (1.30)	
12 hours	10 U	217.64 (31.95)	254.97 (37.44)	222.97 (32.74)	208.87 (30.67)	8.28 (1.22)	250.86
	20 U	346.78 (50.91)	354.02 (51.98)	335.35 (49.24)	311.74 (45.77)	9.09 (1.33)	
	30 U	373.07 (54.77)	376.87 (55.33)	367.73 (53.99)	366.59 (53.82)	8.86 (1.30)	
24 hours	10 U	307.16 (45.10)	327.73 (48.12)	314.78 (46.22)	297.25 (43.64)	8.28 (1.22)	308.09
	20 U	429.83 (63.11)	437.45 (64.22)	402.40 (59.08)	392.12 (57.57)	8.90 (1.31)	
	30 U	434.40 (63.78)	437.83 (64.28)	411.16 (60.37)	403.16 (59.19)	8.89 (1.31)	
48 hours	10 U	310.87 (45.64)	326.21 (47.89)	310.86 (45.64)	296.88 (43.59)	8.66 (1.27)	308.27
	20 U	428.30 (62.88)	438.97 (64.45)	409.26 (60.09)	394.02 (57.85)	8.90 (1.31)	
	30 U	425.26 (62.43)	434.78 (63.83)	409.35 (60.10)	412.68 (60.59)	8.99 (1.32)	
Means for Fungal crude enzymes (B)		**325.88**	**340.40**	**320.05**	**310.27**	**8.71**	
Means enzyme conc. (C)		10 U			202.61		
		20 U			282.97		
		30 U			297.61		
		SEm ±			**CD 1%**		
Treatments (A)		0.517			1.914		
Crude enzymes (B)		0.578			2.139		
Enzyme concentration (C)		0.448			1.657		
A x B		1.156			4.279		
A x C		0.895			3.314		
B x C		1.001			3.706		
A x B x C		2.002			7.411		

Figures in parentheses indicate per cent saccarification
Note : Initial cellulose content 613 mg g^{-1}

T. *reesei* cellulase by cellobiose and glucose during the saccharification. The study indicated the cellulase activity of mere 37 per cent (of its potential) in presence of 9.2 per cent cellobiose and 55 per cent glucose concentration. The release of reducing sugars and per cent saccharification with crude enzymes of saccharolyitic fungi other than *T. reesei* (Tables 2, 3, 4, 5 and 6) was found to be significantly lesser in all the pretreated substrates irrespective of enzyme concentrations.

The trend of release of reducing sugars observed with 20U g^{-1} enzyme concentration with *T. reesei* crude enzyme inoculation was exhibited in all the crude enzyme extracts of different fungi. It was also observed that the release of reducing sugars was significantly highest with higher concentrations of enzyme (30 U) in the initial stages but had reached saturation point in 24 h incubation with release of reducing sugars and found on par with 20 U enzyme concentration in 24 h incubation. These results

Table 5: Effect of different concentrations of crude enzymes on release of reducing sugars in corn husk (mg/g)

Treatments (A)	Enzyme conc. (C)	Fungal crude enzymes (B)					Means for treatments (A)
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowii*	*Aspergillus awamorii*	Control	
6 hours	10 U	152.88 (22.19)	163.54 (23.74)	147.55 (21.42)	138.78 (20.14)	8.28 (1.20)	166.18
	20 U	220.30 (31.98)	229.45 (33.31)	210.40 (30.54)	207.73 (30.15)	8.33 (1.21)	
	30 U	236.68 (34.36)	268.69 (39.00)	237.07 (34.41)	254.21 (36.90)	8.81 (1.28)	
12 hours	10 U	211.54 (30.71)	266.40 (38.67)	207.35 (30.10)	209.64 (30.43)	8.28 (1.20)	240.42
	20 U	317.45 (46.08)	330.78 (48.02)	309.06 (44.86)	305.25 (44.31)	8.71 (1.27)	
	30 U	321.26 (46.64)	380.02 (55.17)	369.54 (53.64)	352.12 (51.11)	8.84 (1.28)	
24 hours	10 U	301.83 (43.81)	323.92 (47.02)	307.54 (44.64)	297.25 (43.15)	8.28 (1.20)	296.33
	20 U	395.92 (57.47)	411.26 (59.70)	381.07 (55.32)	377.26 (54.76)	8.90 (1.29)	
	30 U	410.76 (59.63)	421.44 (61.18)	403.10 (58.52)	387.54 (56.25)	8.89 (1.29)	
48 hours	10 U	303.49 (44.06)	322.78 (46.85)	307.92 (44.70)	298.02 (43.26)	9.04 (1.31)	298.26
	20 U	401.78 (58.32)	416.26 (60.42)	389.83 (56.59)	382.97 (55.59)	9.09 (1.32)	
	30 U	411.21 (59.69)	422.21 (61.29)	401.25 (58.25)	389.07 (56.48)	8.98 (1.30)	
Means for Fungal crude enzymes (B)		**307.09**	**329.73**	**305.97**	**299.99**	**8.70**	
Means enzyme conc. (C)		10 U			199.72		
		20 U			266.09		
		30 U			285.08		
		SEm ±			**CD 1%**		
Treatments (A)		0.578			2.141		
Crude enzymes (B)		0.647			2.393		
Enzyme concentration (C)		0.501			1.854		
A x B		1.293			4.787		
A x C		1.002			3.708		
B x C		1.120			4.146		
A x B x C		2.240			8.291		

Figures in parentheses indicate per cent saccarification
Note : Initial cellulose content 620 mg g^{-1}

suggest that the optimum crude enzyme concentration required for saccharification with crude enzymes of different fungi was 20U g^{-1} substrate for all the pretreated substrates.

CONCLUSION

The inoculation of *T. reesei* crude enzyme extract at 20 U per g concentration along with 5.0% substrate concentration and 24 h incubation was found optimum for higher saccharification. This combination of crude enzymes produced significantly the superior amounts of reducing sugars release and per cent saccharification respectively in sugarcane bagasse (477.45 mg g^{-1} and 67.88%), sugarcane tops (428.68 mg g^{-1} and 62.94%), sugarcane trash (437.45 mg g^{-1} and 64.22%), corn husk (411.26 mg g^{-1} and 59.70%) and in corn stover (421.83 mg g^{-1} and 61.32%). The use of 30 U per g or extension of incubation period up to 48 h did not release significant amount of reducing sugars and stood on par with 20U per g enzyme concentration and 24 h incubation period.

Table 6: Effect of different concentrations of crude enzymes on release of reducing sugars in corn stover (mg/g)

Treatments (A)	Enzyme conc. (C)	Fungal crude enzymes (B)					Means for treatments (A)
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowii*	*Aspergillus awamorii*	Control	
6 hours	10 U	140.31 (20.60)	158.97 (23.34)	141.07 (20.71)	132.30 (19.43)	8.28 (1.22)	163.42
	20 U	210.78 (30.95)	222.97 (32.74)	208.12 (30.55)	209.29 (30.73)	8.52 (1.25)	
	30 U	228.68 (33.58)	259.16 (38.05)	256.11 (37.60)	257.99 (37.88)	8.80 (1.29)	
12 hours	10 U	205.83 (30.22)	257.64 (37.83)	206.21 (30.28)	201.59 (29.60)	8.28 (1.22)	238.90
	20 U	321.64 (47.22)	331.93 (48.73)	310.21 (45.54)	300.31 (44.09)	8.71 (1.28)	
	30 U	335.06 (49.19)	362.40 (53.21)	361.64 (53.10)	363.16 (53.32)	8.86 (1.30)	
24 hours	10 U	298.78 (43.87)	313.26 (45.99)	305.64 (44.87)	287.35 (42.19)	8.66 (1.27)	295.14
	20 U	400.45 (58.79)	421.83 (61.93)	374.95 (55.05)	380.68 (55.89)	8.90 (1.31)	
	30 U	408.50 (59.97)	424.50 (62.32)	393.26 (57.74)	391.40 (57.46)	8.92 (1.31)	
48 hours	10 U	302.24 (44.37)	312.10 (45.82)	303.05 (44.49)	288.87 (42.41)	9.04 (1.33)	294.63
	20 U	403.64 (59.26)	419.24 (61.55)	378.40 (55.55)	385.25 (56.56)	8.90 (1.31)	
	30 U	410.45 (60.26)	421.11 (61.83)	382.21 (56.12)	386.02 (56.67)	8.98 (1.32)	
Means for Fungal crude enzymes (B)		**305.53**	**325.43**	**301.74**	**298.69**	**8.74**	
Means enzyme conc. (C)		10 U				194.47	
		20 U				265.74	
		30 U				283.86	
		SEm ±				**CD 1%**	
Treatments (A)		0.453				1.678	
Crude enzymes (B)		0.507				1.876	
Enzyme concentration (C)		0.393				1.453	
A x B		1.014				3.752	
A x C		0.785				2.906	
B x C		0.878				3.249	
A x B x C		1.756				6.498	

Figures in parentheses indicate per cent saccarification
Note : Initial cellulose content 613 mg g^{-1}

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