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SACCHARIFICATION OF *Leptochloa fusca* (KALLAR GRASS STRAW) USING THERMOSTABLE CELLULASES

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Abstract

Saccharifying ability of thermostable cellulases on kallar grass straw at 50 and 60°C was evaluated. Enzyme filtrate from Chaetomium thermophile *saccharified 5% kallar grass straw to 69% reducing sugars (quantitatively) at 50°C. Culture filtrates from all the thermophilic fungi showed a considerable saccharification rate up to 40 h. Freeze-dried enzyme of* Trichoderma reesei *VTT-D-79125 (mesophile), even at higher enzyme concentration resulted in 60% reducing sugars yield (quantitatively). Glucose concentration in the hydrolysates from the fungi was in the order of* C. thermophile > T. reesei > Sporotrichum thermophile > Aspergillus fumigatus > Torula thermophila > Humicola grisea > Malbranchea pulchella. *At 60°C, thermostable enzymes hydrolysed kallar grass straw at a maximum rate for the initial 20 h, after which the rate essentially stopped increasing. However, at elevated temperature* C. thermophile *enzymes saccharified kallar grass straw up to 20 h to almost the same yield in half the time as compared to that at 50°C. The overall decline in saccharification yield (11%) of* C. thermophile *enzymes was significantly lower than* T. reesei *enzymes (34%) at 60°C.*

Key words: Saccharification, lignocellulose, kallar grass straw, thermostable, cellulases.

INTRODUCTION

The economics of enzymatic hydrolysis of lignocellulosic substrates can be improved by increased saccharification rates and reduced cost of enzyme production (Allen *et al.*, 1984). In a previous study (Latif, 1992) it was revealed that indigenous thermophilic fungal isolates produce cellulases/xylanases from untreated kallar grass straw. These enzymes can tolerate higher temperatures than enzymes from mesophilic fungi. Thermophilic microorganisms are reported to produce enzymes which show thermostability at temperatures of 70–80°C (Margaritis & Merchant, 1983, 1986). Thermostable xylanases have a commercial application in the bleach boosting of wood pulps, which leads to a higher final brightness (Zamost *et al.*, 1991). Temperature also plays an important role in the cellulolysis of the substrate. However, there are only a few reports on successful saccharification at temperatures higher than 50°C (Skinner & Tokuyama, 1978; Ekhlund *et al.*, 1990). Use of thermostable enzymes is also increasing, partly due to the ability to clone thermophilic genes into mesophilic hosts (Fujii *et al.*, 1983; Sen & Oriel, 1989).

This study deals with the hydrolysis of alkali-pretreated kallar grass straw at elevated temperatures using enzyme preparations from thermophilic fungi and *T. reesei* mutant VTT-D-79125. Products of hydrolysis were evaluated by HPLC.

METHODS

Substrate pretreatment

Kallar grass straw was harvested from the Bio-saline Research Station (BSRS) in Lahore, Pakistan, during summer. The substrates were dried to constant weight at 80°C in an oven before milling to 0·5 mm particle size. The milled substrates were kept at room temperature. Sodium hydroxide (2%) treatment with autoclaving was according to Latif *et al.* (1988). The present pretreated substrate had the following composition as percentage of dry weight: lignin 10·3; polysaccharides 77 (cellulose 64·5 and hemicellulose 12·5).

Cellulase sources

Commercial enzyme preparation included freeze-dried cellulases from *T. reesei* mutant strain VTT-D-79125 (from BfH, Hamburg, Germany). The enzyme source was found to contain 1·0 and 0·71 U/mg (enzyme powder) of FP-ase and β-glucosidase activities detected according to Saddler *et al.* (1985), a slight modification of Mandel *et al.* (1976) and Rajoka and Malik (1986). Culture filtrates of thermophilic fungi were obtained after growing test organisms on 4% kallar grass straw. Enzyme titres of these fungi had the following FP-ase and β-glucosidase activities, respectively, in U/ml: *A. fumigatus*, 0·5, 0·65; *C. thermophile*, 0·35, 0·52; *H. grisea*, 0·30, 0·64; *S. thermophile*, 0·5, 0·70; *T. thermophila*, 0·5, 0·64; and *M. pulchella*, 0·3, 0·08.

Enzymatic saccharification

Enzymatic hydrolysis of pretreated kallar grass straw (5%) was carried out at 50 and 60°C. Enzyme culture

filtrate (20 ml) from thermophilic fungi, adjusted to pH 5·0 was used in 100 ml conical flasks (in duplicate) at 120 rpm, for 48 h or more. Predetermined enzyme activities of these culture filtrates had the following titre of FP-ase and β-glucosidase, respectively, in U/g substrate: *A. fumigatus*, 10·0, 13·0; *C. thermophile*, 7·0, 10·4; *H. grisea*, 6·0, 12·8; *S. thermophile*, 10·0, 13·6; *T. thermophila*, 10·0, 12·8; and *M. pulchella*, 0·6, 1·6. Cellulase powder from *T. reesei* VTT-D-79125 prepared in citrate buffer (0·05 M, pH 5·0) had the enzyme dosage of FP-ase: β-glucosidase of 30:21 U/g substrate.

Sugar analysis

At different time intervals, 0·5 ml of samples were withdrawn from the hydrolysates (taking care that the slurry density was not disturbed) and were centrifuged for 1–2 min at 3000 rpm. Reducing sugars in the supernatant were determined according to Miller (1959).

Sugar composition was determined by HPLC (Gilson Co., France) using a cation exchange column of aminex HPX-87H running 0·001 N H_2SO_4 as eluent. A flow rate of 0·6 ml/min was maintained at a column temperature of 85°C. Suitably diluted samples (20 μl) were injected through a rheodyne injection valve loop. Standard sugars of D-glucose, D-xylose and cellobiose (GLC grade) and samples were detected on a refractive index detector (Shimadzu Co., Japan).

Saccharification yields were calculated quantitatively using the equation:

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars (mg/ml)} \times 0{\cdot}9 \times 100}{\text{Substrate (Polysaccharides) (mg/ml)} \times 0{\cdot}77}$$

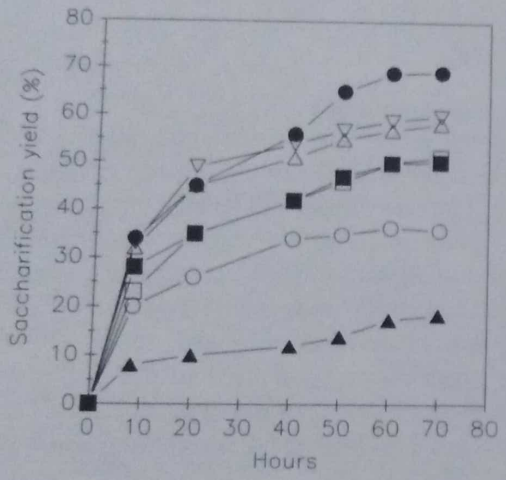

Fig. 1. Time course saccharification of 5% kallar grass straw using cellulase preparations from fungi at 50°C. *A. fumigatus* (○); *C. thermophile* (●); *H. grisea* (△); *S. thermophile* (□); *T. thermophila* (■); *M. pulchella* (▲); *T. reesei* VTT-D-79125 (▽). Standard deviations of triplicate values were in the range 2–3%.

RESULTS

Saccharification at 50°C

The yield of reducing sugars increased rapidly during the first 10 h (Fig. 1) of saccharification. During 10–50 h the rate slowed down considerably, after which the increase was minimal except for *C. thermophile*, which showed a saccharification yield of 69% sugars (quantitatively) up to 70 h. Saccharification yield from various enzyme preparations was in the following order: *C. thermophile* > *T. reesei* VTT-D-125 > *H. grisea* > *S. thermophile* > *T. thermophila* > *A. fumigatus* > *M. pulchella*.

The composition of sugars in the hydrolysates as determined by HPLC (Table 1) showed various sugar components, with different enzyme preparations. The three main sugar components detected were glucose, cellobiose and xylose, while the remaining three peaks were pooled as oligosaccharides. In the hydrolysates produced by these fungi, glucose was the main sugar component, except for *M. pulchella*, which showed higher xylose content. The enzyme filtrate of *C. thermophile* produced the highest glucose yield. Xylose was found to be present in considerable amounts in the hydrolysates of these fungi except *S. thermophile* and *A. fumigatus*, which showed greater amounts of oligosaccharides in the hydrolysates compared to that from *T. reesei*. Except for *M. pulchella* and *T. reesei*, the other fungi also produced a small amount of cellobiose.

Sugar composition in the hydrolysates, calibrated in terms of percentage relative yield of each component,

Table 1. Comparison of saccharification yield from 5% kallar grass straw by various cellulase sources at 50°C for 70 h

Enzyme source	Sugar yield (%)				
	Reducing sugars	Cellobiose	Glucose	Xylose	Oligosaccharide
A. fumigatus	36·5	5·6	12·3	3·2	15·4
C. thermophile	69·2	5·3	31·6	18·8	13·5
H. grisea	58·4	4·0	23·3	17·6	13·5
M. pulchella	18·7	0·0	3·6	6·8	8·3
S. thermophile	51·4	2·7	24·5	2·0	22·2
T. thermophila	50·3	5·7	18·2	15·2	11·2
T. reesei	60·0	0·0	28·5	11·6	20·0

Standard deviations of assays in triplicate were in the range of 2–3%.

is shown in Fig. 2 (see Methods). Higher levels of glucose were found in the hydrolysates from *S. thermophile*, *T. reesei* and *C. thermophile*. The latter two, along with *H. grisea*, *T. thermophila* and *M. pulchella*, also showed higher levels of xylose. On the other hand, *A. fumigatus*, *M. pulchella* and *S. thermophile* showed higher levels of oligosaccharides. The cellobiose concentration was comparatively higher from *A. fumigatus* and *T. thermophila* enzyme preparations.

Saccharification at 60°C

Enzyme filtrates (as in the previous experiment) were used to saccharify 5% kallar grass straw at 60°C. A time course study revealed that at 60°C the rate of reaction was almost maximum up to 20 h, except for *C. thermophile* and to some extent for the *T. thermophila* enzyme source (Fig. 3). Maximum saccharification yield of 58% sugars (quantitatively) was obtained from *C. thermophile* enzymes (Table 2). Although maximum yields for thermostable enzyme preparations were attained at almost half the time as compared to 50°C, the overall saccharification yields were significantly lower. However, at 60°C, the saccharification yield of *C. thermophile* was decreased by only 11%, as compared to a decrease of 34% from *T. reesei* VTT-D-75125 (mesophile).

The percent glucose in the hydrolysates was greater for *T. reesei*, *C. thermophile* and *H. grisea* (Fig. 4). The latter two along with *T. thermophila* showed similar levels of relative xylose content. Greater levels of oligosaccharides were found to be present in the hydrolysates of *A. fumigatus*, *S. thermophile* and *M. pulchella*.

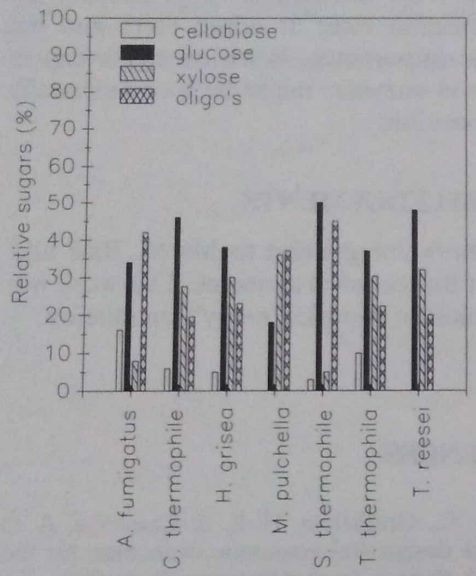

Fig. 2. Relative percentage yield of different sugars in the hydrolysates at 50°C as determined by HPLC. Standard deviations of assays in triplicate were in the range 2–3%.

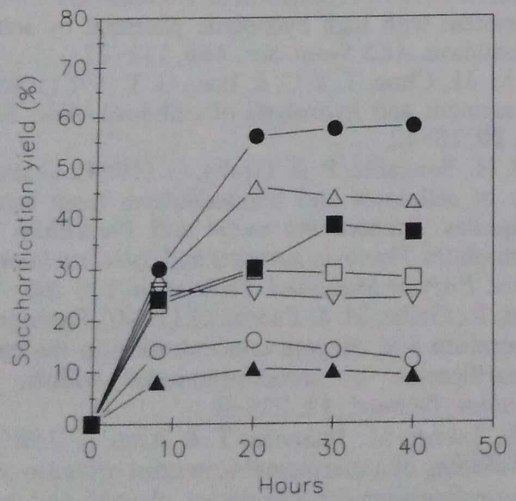

Fig. 3. Time course saccharification of 5% kallar grass straw using cellulase preparations from fungi at 60°C. *A. fumigatus* (○); *C. thermophile* (●); *H. grisea* (△); *S. thermophile* (□); *T. thermophila* (■); *M. pulchella* (▲); *T. reesei* VTT-D-79125 (▽). Standard deviations of triplicate values were in the range 2–3%.

DISCUSSION

The feasibility of lignocellulose bioconversion largely depends upon process economics. Cost of enzymes is a major constraint. Saccharification of kallar grass straw at 50 and 60°C by thermostable dilute enzymes resulted in considerable saccharification up to 50 h and 20 h, respectively. Among the various enzyme sources used culture filtrate of *Chaetomium thermophile* saccharified kallar grass straw (5%) to 69% and 58% reducing sugars (quantitatively) after 70 h and 40 h, respectively. Saccharification rate slowed down after 10 h, which may be explained by the increase in crystalline portion of the substrate as reported by Van Dyke (1972) and Chang *et al.* (1981). Van Dyke (1972) described the rates as first order with respect to amorphous, crystalline and resistant substrates. Thermostable cellulase from these indigenous fungi showed significantly higher saccharification rates as compared to *T. reesei* VTT-D-79125 at elevated temperature. This fact is attributed to the increased half-life of these cellulases as depicted by the stability of FP-ase at elevated temperature up to 70°C (Latif, 1992). *T. reesei* cellulases were active up to 10 h because of lower enzyme stability of mesophiles, which agrees with Ekhlund *et al.* (1990). The initial saccharification rate of thermophilic fungi at 60°C increased up to 20 h, then became constant. *C. thermophile* cellulases showed increased saccharification rate up to 40 h. In fact, *C. thermophile* yielded almost 1·4 times more sugars up to 20 h at 60°C than that at 50°C. Durand *et al.* (1984) using *Thielavia terrestris* (NRRL 8126) at 60°C obtained glucose yields of 52% and 2·9% from cellulose powder and bagasse, respectively. Bisaria and Ghose (1978) showed that maximum level of adsorption decreased with an increase in temperature after 50°C. β-Glucosidase appears to be more susceptible to high temperatures, whereas presence of substrate provides some protection (Mandels & Reese, 1964; Stoppok *et al.*, 1982; Durand *et al.*, 1984).

Table 2. Comparison of saccharification yield from 5% kallar grass straw by various cellulase sources at 60°C for 40 h

Enzyme source	Sugar yield (%)				
	Reducing sugars	Cellobiose	Glucose	Xylose	Oligosaccharide
A. fumigatus	15·9	0·0	3·4	1·9	10·5
C. thermophile	58·0	5·5	23·2	18·2	13·2
H. grisea	46·0	2·1	14·2	14·7	14·3
M. pulchella	10·5	2·5	0·9	3·2	3·9
S. thermophile	29·5	0·0	8·3	2·0	19·6
T. thermophila	38·5	2·3	10·1	13·9	12·2
T. reesei	26·0	0·0	11·9	8·1	6·0

Standard deviations of assays in triplicate were in the range of 2–3%.

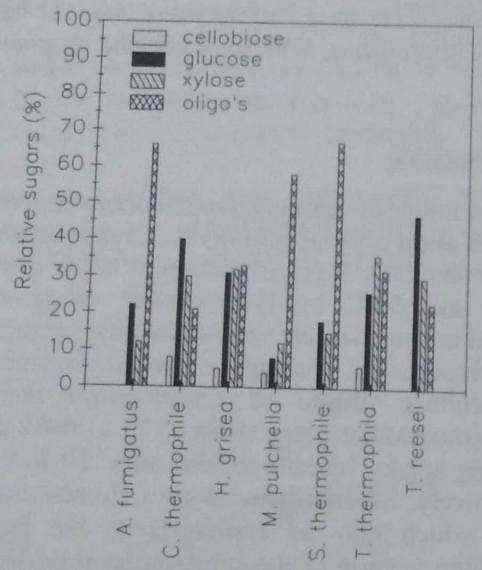

Fig. 4. Relative percentage yield of different sugars in the hydrolysates at 60°C as determined by HPLC. Standard deviations of assays in triplicate were in the range 2–3%.

Relative yields of different sugars in the hydrolysates indicate that *S. thermophile*, and to some extent *A. fumigatus*, show quantitatively greater hexose levels, relative to other sugars because of their higher levels of β-glucosidase (Latif, 1992). The ratio of 1:1·5 of FP-ase:β-glucosidase is an important parameter as far as saccharification for ethanol production is concerned (Ghose & Ghosh, 1990; Chahal, 1991). In contrast, the other enzyme sources showed higher levels of pentoses, indicating greater xylanase levels. Thus, fermentation process by a yeast, which grows on hexoses or both hexoses and pentoses for biofuel production is feasible.

Decline in the percentage glucose, in contrast to xylose level, in the hydrolysates at higher temperatures could be attributed to a more pronounced inactivation of their β-glucosidase component.

CONCLUSION

Kallar grass straw can be used as a lignocellulosic substrate for biotechnological applications, such as saccharification into fermentable sugars. Thermostable cellulases from indigenous fungi showed significant saccharification rates at dilute titers and can be of economic importance. Work on hyper-expression of these novel enzymes might be a commercially attractive proposition.

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